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ORIGINAL RESEARCH

Macrophage Polarization-Related Biomarkers in Epilepsy: Integrated Bioinformatics Analysis and Clinical Validation

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Purpose: Accumulating evidence implicates macrophage polarization in the pathogenesis of epilepsy. This study integrated bioinformatics and experimental approaches to identify macrophage polarization-related genes (MPRGs) as potential biomarkers and to elucidate their putative causal roles and immune associations in epilepsy.

Patients and Methods: Publicly available transcriptomic datasets from the Gene Expression Omnibus (GEO; GSE143272, GSE88992, and GSE201048) were analyzed. Macrophage polarization-related biomarkers with diagnostic potential were identified via integrated analyses, including immune infiltration, weighted gene co-expression network analysis (WGCNA), differential expression, machine learning algorithms, expression profiling, and receiver operating characteristic (ROC) curves. Two-sample Mendelian randomization (MR) was performed to assess the causal effects on epilepsy. Biomarker-immune cell associations and single-cell expression patterns were analyzed, and biomarker expression was validated at transcriptional (mRNA) and protein levels in clinical samples.

Results: Integrated analyses identified four macrophage polarization-related candidate biomarkers for epilepsy—DNAH1, PGRMC2, RFX7, and TFRC—all exhibiting strong diagnostic performance. Two-sample MR suggested a putative protective causal association between genetically predicted DNAH1 expression and epilepsy risk (OR < 1, P < 0.05). All four biomarkers showed negative associations with macrophage infiltration, and single-cell analysis demonstrated their cellular distribution patterns, with DNAH1 and PGRMC2 mainly detected in astrocytes, RFX7 in T cells, and TFRC in microglia. Among the four candidate biomarkers identified through integrated bioinformatics analyses, DNAH1 and TFRC were further validated in peripheral blood samples at both the transcriptional and protein levels.

Conclusion: This study identified macrophage polarization–related candidate biomarkers associated with epilepsy through integrated bioinformatics analyses. Among them, DNAH1 and TFRC were supported by clinical blood-based validation, with DNAH1 showing a putative protective causal role, highlighting their potential translational relevance in epilepsy.

Keywords: epilepsy, macrophage polarization, biomarkers, immune microenvironment, mendelian randomization

Introduction

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and a persistent predisposition to further attacks. It profoundly affects patients' quality of life, particularly among those with drug-resistant forms, for whom accurate diagnosis and effective treatment remain major clinical challenges.[1] In 2017, the International League Against Epilepsy (ILAE) introduced a new diagnostic framework that categorizes etiologies into six major groups and formally incorporates

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comorbidities—such as anxiety and sleep disturbances—into diagnostic considerations.[2] While this framework has refined diagnostic precision and encouraged deeper investigation into disease mechanisms, the overall global burden of epilepsy remains substantial. According to the World Health Organization, approximately 50 million individuals worldwide were living with epilepsy in 2024, nearly 80% of them in low- and middle-income countries. This prevalence imposes immense psychological and economic stress on patients and their families while straining healthcare systems worldwide.[3] Consequently, early and accurate diagnosis remains essential to improving patient outcomes and quality of life.

Growing evidence suggests that neuroimmune inflammation and oxidative stress are central to the pathogenesis of many central nervous system disorders, including epilepsy.[4] In the brain, microglia—the resident macrophages of the central nervous system—undergo phenotypic polarization following injury.[5] This process mediates a dynamic shift between pro-inflammatory and reparative states that helps restore the balance between neuroinflammation and tissue repair. Pro-inflammatory polarization, for instance, activates the NLRP3 inflammasome through IL-1β release, disrupts the blood–brain barrier, and recruits peripheral monocytes via the CCL2–CCR2 axis. These cascades collectively enhance neuronal excitability.[6,7] During status epilepticus (SE), seizure-induced damage-associated molecular patterns such as HMGB1 further amplify inflammatory responses through the NF-κB/MAPK pathway, thereby lowering the seizure threshold and exacerbating neuronal damage.[8] Understanding how macrophage polarization shapes these immune processes and influences neuronal network activity is therefore crucial for clarifying epileptogenic mechanisms and developing targeted anti-inflammatory interventions.

Therefore, we systematically identified key biomarkers linked to macrophage polarization in epilepsy by integrating multi-omics bioinformatic analyses with clinical validation. We then examined the functional roles of these biomarkers through enrichment, immune infiltration, and single-cell analyses. Our findings shed light on neuroimmune mechanisms underlying epilepsy and highlight potential diagnostic and therapeutic targets for future translational research.

Materials and Methods

Data Acquisition

Three epilepsy datasets were obtained from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), including two bulk RNA-seq datasets (GSE143272 and GSE88992) and one single-cell RNA-seq dataset (GSE201048). GSE143272 (Platform: GPL10558) comprised 51 control and 34 epilepsy peripheral blood samples and was used as a training cohort.[9] GSE88992 (Platform: GPL1261) included 9 control and 8 epilepsy hippocampal samples from a mouse model of mesial temporal lobe epilepsy, serving as a validation set.[10] The single-cell dataset, GSE201048 (Platform: GPL18573), contained 11 epilepsy brain tissue samples.[11] Additionally, a total of 35 macrophage polarization-related genes (MPRGs) were extracted from the MSigDB database (https://www.gsea-msigdb.org/gsea/msigdb).[12]

Immune Infiltration Analysis

To evaluate differences in immune cell infiltration between epilepsy and control groups, the single-sample gene set enrichment analysis (ssGSEA) algorithm implemented in the "GSVA" R package (version 1.46.0)[13] was applied to estimate the relative enrichment scores of 28 immune cell types. The distribution of immune cell infiltration was visualized as a stacked bar chart using the "ggplot2" R package (version 3.5.1). Differences in immune cell infiltration between groups were assessed using the Wilcoxon rank-sum test, with $P < 0.05$ considered statistically significant.

Weighted Gene Co-Expression Network Analysis (WGCNA)

The ssGSEA algorithm was used to calculate the MPRG score for each sample, and differences between the epilepsy and control groups were analyzed. Using the MPRG score as a phenotypic trait, WGCNA was then performed. After removing outlier samples, the optimal soft-thresholding power (β) was selected to construct a scale-free co-expression network. Subsequently, a hierarchical clustering dendrogram was generated based on topological overlap, with the minimum module size set to 100 using the hybrid dynamic tree-cutting algorithm. Pearson correlation analysis was applied to evaluate relationships between gene modules and the MPRG score. The module with the highest absolute correlation coefficient was identified as the key module, and its constituent genes were defined as module genes.

Differentially Expressed Analysis

Differentially expressed genes (DEGs) between epilepsy and control groups were discovered via the "limma" R package (version 3.54.2), with thresholds of $|\log_2 FC| > \log_2(1.2)$ and $P < 0.05$.[14] Results were shown through a volcano plot and a heatmap generated with the "ggplot2" (version 3.5.1) and the "pheatmap" (version 1.0.12) R packages, respectively. The intersection of DEGs and module genes was then identified, and the overlapping genes were defined as candidate genes for subsequent analyses.

Functional Enrichment Analysis

The clusterProfiler R package (version 4.6.2) was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the candidate genes to elucidate their potential biological functions.[15] GO terms and KEGG pathways with adjusted $P < 0.05$ were considered significantly enriched.

Development of the Protein-Protein Interaction (PPI) Network

To further examine interactions among candidate genes at the protein level, the STRING database (https://string-db.org/) was utilized to create the PPI network. The results were uploaded to the Cytoscape software (version 3.10.2), and the PPI network was visualized.[16]

Identification of Macrophage Polarization-Related Biomarkers for Epilepsy

Three machine learning algorithms — least absolute shrinkage and selection operator (LASSO) regression, support vector machine-recursive feature elimination (SVM-RFE), and Boruta — were applied to candidate genes to identify those most associated with MPRGs in epilepsy. These analyses were implemented using the "glmnet" (version 4.1–8),[17] "e1071" (version 1.7–14), and "Boruta" (version 8.0.0) R packages, respectively. The characteristic genes identified by all three algorithms were intersected to obtain overlapping candidates. Subsequently, the expression patterns and diagnostic performance of these genes were evaluated in both GSE143272 and GSE88992. Genes showing consistent expression trends and an area under the receiver operating characteristic (ROC) curve (AUC) greater than 0.7 in both datasets were defined as biomarkers for epilepsy. Furthermore, correlations among the identified biomarkers were analysed using Spearman algorithm, and their chromosomal locations were visualized using the "RCircos" R package (version 1.2.2).[18]

Development and Assessment of Nomogram

To predict epilepsy risk, the nomogram was created on the basis of the expression of biomarkers via the "rms" R package (version 6.8–0). In the nomogram, each gene was assigned a specific point value, and the total points represented the aggregate score of all included genes. The scoring system transformed complex genomic data into an intuitive graphical format to assist clinical decision-making. The predictive performance of the nomogram was further evaluated using calibration and receiver operating characteristic (ROC) curves.

Two-Sample Mendelian Randomization (TSMR) Analysis

To investigate the causal effects of the identified biomarkers on epilepsy, the TSMR analysis was conducted by the "TwoSampleMR" R package (version 0.6.3). Genome-wide association study (GWAS) summary data for the biomarkers was obtained from the eQTLGen database (https://www.eqtlgen.org/):

- TFRC (eqtl-a-ENSG00000072274; 26,609 samples, 20,304 SNPs),
- DNAH1 (eqtl-a-ENSG00000114841; 31,684 samples, 16,442 SNPs),
- PGRMC2 (eqtl-a-ENSG00000164040; 31,470 samples, 17,112 SNPs), and
- RFX7 (eqtl-a-ENSG00000181827; 26,196 samples, 18,658 SNPs).

GWAS data for epilepsy were derived from the FinnGen R12 database, including two datasets: finngen_R12_GE (generalized epilepsy; 1690 cases and 484,697 controls) and finngen_R12_GE_STRICT (generalized epilepsy, strict definition; 873 cases and 484,697 controls).

Instrumental variables (IVs) were selected according to the three core assumptions of MR analysis using the extract_instruments() function in "TwoSampleMR". SNPs were retained if they met the following criteria:

1. Strong association with the exposure ($P < 5\times10\text{-}8$);
2. Independence from linkage disequilibrium (LD; r2 = 0.01, distance = 10,000 kb);
3. F-statistic > 10; and
4. No significant associations with the outcome.

TSMR analysis was conducted using the mr() function with five complementary methods: MR Egger, Weighted median, Inverse variance weighted (IVW), Simple mode, and Weighted mode. The IVW estimates were regarded as the primary results. To evaluate the robustness of the causal inference, heterogeneity test, horizontal pleiotropic test, and Leave-one-out (LOO) analyses were performed. In addition, the directionality of causality was examined using the Steiger test.

Gene Set Enrichment Analysis (GSEA)

To understand possible mechanisms of biomarkers involvement in epilepsy, GSEA was conducted by the "clusterProfiler" R package (version 4.6.2) (screening criteria: |NES| > 1 and adjusted.$P < 0.05$). The "c2.cp.kegg_medicus.v2023.2.Hs.symbols.gmt" was extracted from the MSigDB database (http://www.gsea-MSigdb.org/gsea/msigdb) as the reference set. The associations between biomarkers and all genes were evaluated using Spearman correlation and ranked according to the correlation coefficients.

Drug Prediction

To find novel potential targets and more effective drugs, the DSIGDB database (https://dsigdb.tanlab.org/DSigDBv1.0/) was applied to forecast potential drugs with biomarkers as targets (adjusted.$P < 0.05$). The results were uploaded to the Cytoscape software (version 3.10.2), and the mRNA-drug interaction network was displayed.

Single-Cell Analysis

To investigate biomarker expression at the cellular level, raw data from GSE201048 was obtained from the GEO database. Quality control (QC) was conducted by the "seurat" R package (version 5.1.0) to obtain high-quality cells and genes. The filtering criteria were as follows: (1) cells expressing between 200 and 4000 genes were retained; (2) genes detected in 200 to 20,000 counts were retained; (3) genes expressed in fewer than 3 cells were excluded; and (4) cells with the mitochondrial gene proportions exceeding 20% were removed. Data were then normalized using SCTransform(), and the top 2000 highly variable genes(HVGs) were identified with the VST method in the FindVariableFeatures() function. Dimensionality reduction was performed by principal component analysis (PCA), and the top 30 principal components (PCs) were selected based on the elbow plot for downstream analyses. Cell clustering was conducted with the FindNeighbors() and FindClusters() functions (resolution = 0.5), and the results were visualized using UMAP. Cell types were annotated by comparing HVGs in each cluster with known marker genes reported in the literature.[11,19] Furthermore, the relative proportions of each cell type were calculated, and the expression of biomarker in each cell type was analyzed. Additionally, to reveal the interactions among cell types, Cellchat (version 1.6.1) was applied to construct the cellular communication analysis.

Sample Collection

Peripheral blood samples were collected from 7 patients with epilepsy and 6 healthy controls at the First Hospital of Shanxi Medical University between March and April 2025. The inclusion criteria were as follows: (1) age between 18 and 80 years; (2) diagnosis of epilepsy according to the 2017 International League Against Epilepsy (ILAE) classification; and (3) current regular treatment with antiseizure medications (ASMs). The exclusion criteria were as follows: (1) severe damage to vital

organs or unstable vital signs; (2) a history or presence of status epilepticus; (3) presence of paroxysmal symptoms requiring differentiation from epilepsy (syncope, transient ischemic attack); (4) pregnancy; and (5) refusal of informed consent by patients and/or legal guardians. This study was approved by the Ethics Committee of the First Hospital of Shanxi Medical University (KYLL-2025-066), and written informed consents were obtained from patients and their legal guardians.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated employing the FastPure Complex Tissue/Cell Total RNA Isolation Kit (RC113-01, Vazyme, China), and RNA purity and concentration were determined with a Nano-500 Micro-Spectrophotometer. According to the instructions of ABScript III RT Master Mix for RT-qPCR with gDNA Remover (RK20429, ABclonal, China), RNA was reverse transcribed into cDNA. RT-qPCR was conducted to examine the expression of biomarkers using the Genious 2X SYBR Green Fast RT-qPCR Mix (RK21205, ABclonal, China), and the expression of biomarkers was quantified using the $2^{-\Delta\Delta ct}$ method. The primer sequences were presented in Table 1.

Western Blotting (WB)

Proteins were extracted from peripheral blood samples using a standard lysis buffer supplemented with protease inhibitors. Protein concentrations were determined using a BCA protein assay kit (P0010, Beyotime, China). Equal amounts of protein were denatured and separated by SDS–PAGE and subsequently transferred onto PVDF membranes (0000279048, Millipore, USA). The membranes were blocked with 5% skim milk at room temperature and incubated with primary antibodies at 4°C overnight, including anti-DNAH1 (Invitrogen, PA5-115132, 1:500), anti-PGRMC2 (Abcam, ab251875, 1:1000), anti-TFRC (Abclonal, A25900, 1:500), and anti-β-actin (Proteintech, 66009-1-Ig, 1:10,000).

After washing, membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Abcam, ab205718, 1:5000). Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate (Thermo Scientific, 34075) and imaged with a chemiluminescence detection system. Band intensities were quantified using ImageJ software and normalized to β-actin. Protein-level validation of RFX7 was not performed due to the lack of a reliable antibody.

Statistical Analysis

R software (version 4.2.2) and GraphPad Prism were applied to conduct all statistical analyses. The disparities were analyzed via the Wilcoxon test. A statistically significant P-value is less than 0.05.

Results

Significant Difference in Macrophage Infiltration Density Among Epilepsy and Control Cohorts

To elucidate the disparity in infiltration abundance of immune cells between epilepsy and control groups, immune infiltration analysis was performed. The stacked bar chart illustrated the infiltration abundance of each immune cell in

Table 1 The Primer Sequences of Biomarkers for RT-qPCR

Gene	Primer Sequences (5'-3')
H-GAPDH	F:5'-GGAGTCCACTGGCGTCTTCA-3' R:5'-GTCATGAGTCCTTCCACGATACC-3'
DNAH1	F:5'-GCCCAGACTGACTTCCCAC-3' R:5'-TCGATCTCAATCTTGCGAGGAT-3'
PGRMC2	F:5'-ATTTGCTGGTAGGGATGCCTC-3' R:5'-TCGAACACTCTCCATTTGTACTG-3'
RFX7	F:5'-CATGAAGGCACGTCGTTTGG-3' R:5'-ACGGTGTCAAATGGTTGGCT-3'
TFRC	F:5'-ACCATTGTCATATACCCGGTTCA-3' R:5'-CAATAGCCCAAGTAGCCAATCAT-3'

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each sample in epilepsy and control groups (Figure 1A). The infiltration abundance of activated CD8 T cells, activated CD4 T cells, follicular helper T cells (Tfh), natural killer T cells, activated dendritic cells, macrophages, eosinophils, mast cells, monocytes, and neutrophils was considerably different between epilepsy and control groups (Figure 1B). The marked difference in infiltration abundance of macrophages indicated the correlation between macrophages and the occurrence of epilepsy, and the MPRGs score between epilepsy and control groups was markedly different (Figure 1C). Given these, we further conducted WGCNA with the MPRGs score as the trait (Figure 1D and E). The MEblue module had the highest absolute value of the correlation coefficient (cor = 0.48, P = 4e-06), and this module was the key module. Moreover, a total of 464 module genes were obtained.

Identification and Enrichment Analysis of Prospective Genes

Differential expression analysis discovered 956 DEGs between epilepsy and control groups, including 509 up-regulated and 447 down-regulated genes (Figure 2A). The top 20 up-regulated and down-regulated genes were displayed through the heatmap (Figure 2B). After the intersection between DEGs and module genes, 52 candidate genes were obtained (Figure 2C). Enrichment analysis revealed that these genes were significantly enriched in 59 GO items and 3 KEGG pathways, such as "ribonucleoprotein complex biogenesis (GO-BP)," "preribosome (GO-CC)," "snoRNA binding (GO-MF)," "Ribosome biogenesis in eukaryotes (KEGG)," "Hematopoietic cell lineage (KEGG)," and "C-type lectin receptor signaling pathway (KEGG)" (Figure 2D and E). At the protein level, the PPI network included 34 nodes and 65 edges (Figure 2F). For instance, BMS1 interacted with ten proteins, including NOL11, IMP4, and DHX37.

Determination of Biomarkers

Using three algorithms, we obtained 6 characteristic genes (LASSO), 47 characteristic genes (SVM-RFE), and 10 characteristic genes (Boruta), respectively (Figure 3A–C). Following the intersection, DNAH1, FAM50A, PGRMC2, RFX7, RPF2, and TFRC were intersection characteristic genes (Figure 3D). In both GSE143272 and GSE88992, the expression levels of DNAH1, PGRMC2, RFX7, and TFRC were markedly decreased in epilepsy, with AUC values for these four genes exceeding 0.7, indicating their strong diagnostic potential for epilepsy (Figure 3E and F). Therefore, DNAH1, PGRMC2, RFX7, and TFRC were identified as macrophage polarization–related candidate biomarkers for epilepsy based on integrated bioinformatics analyses. Chromosome localization analysis illustrated that DNAH1 and TFRC were located at Chromosome 3, PGRMC2 was located at Chromosome 4, and RFX7 was located at Chromosome 15 (Figure 3G). Correlation analysis demonstrated that there were considerably positive correlations among biomarkers, with the highest correlation between RFX7 and PGRMC2 (cor = 0.704) (Figure 3H).

The Causal Influence of Biomarkers on Epilepsy

To understand the causal relationships between biomarkers and epilepsy, TSMR analysis was performed. After selecting IVs, since the numbers of IVs of RFX7 and PGRMC2 were less than 3, we investigated the potential causal association between DNAH1 and epilepsy and between TFRC and epilepsy using Mendelian randomization. As shown in Figure 4A, genetically predicted DNAH1 expression was significantly associated with a reduced risk of epilepsy (outcome: finngen_R12_GE: P = 0.021, OR = 0.559, 95% CI = 0.341–0.918; finngen_R12_GE_STRICT: P = 0.018, OR = 0.438, 95% CI = 0.220–0.870). Moreover, scatter plots, forest plots, and funnel plots further supported these findings (Figure 4B–D). Sensitivity analysis illustrated that heterogeneity and horizontal pleiotropy did not exist (Table 2), and removing a specific SNP had no effect on the MR results (Figure 4E), indicating that MR results were robust.

Construction of a Nomogram for Predicting Epilepsy

To enhance clinical applicability, a nomogram was constructed by integrating the expression levels of the identified biomarkers (Figure 5A). The ROC curve indicated that the AUC value of the nomogram was 0.847 (Figure 5B). The calibration curve suggested that the predicted probability was close to the actual probability (Figure 5C). These results revealed the accuracy and robustness of the nomogram in predicting the occurrence of epilepsy.

Figure 1 Immune infiltration analysis. (**A**) Stacked bar chart showing the infiltration abundance of 28 immune cell types in each sample. (**B**) Box plots indicating differences in immune cell infiltration abundance between epilepsy and control groups. (**C**) Comparison of macrophage polarization-related gene (MPRG) scores between epilepsy and control groups. (**D** and **E**) Weighted Gene Co-expression Network Analysis (WGCNA). (**D**) Determination of the optimal soft-thresholding power. (**E**) Heatmap illustrating correlations between gene modules and MPRG scores. *P < 0.05, **P < 0.01, ****P < 0.0001.
Abbreviation: ns, not significant.

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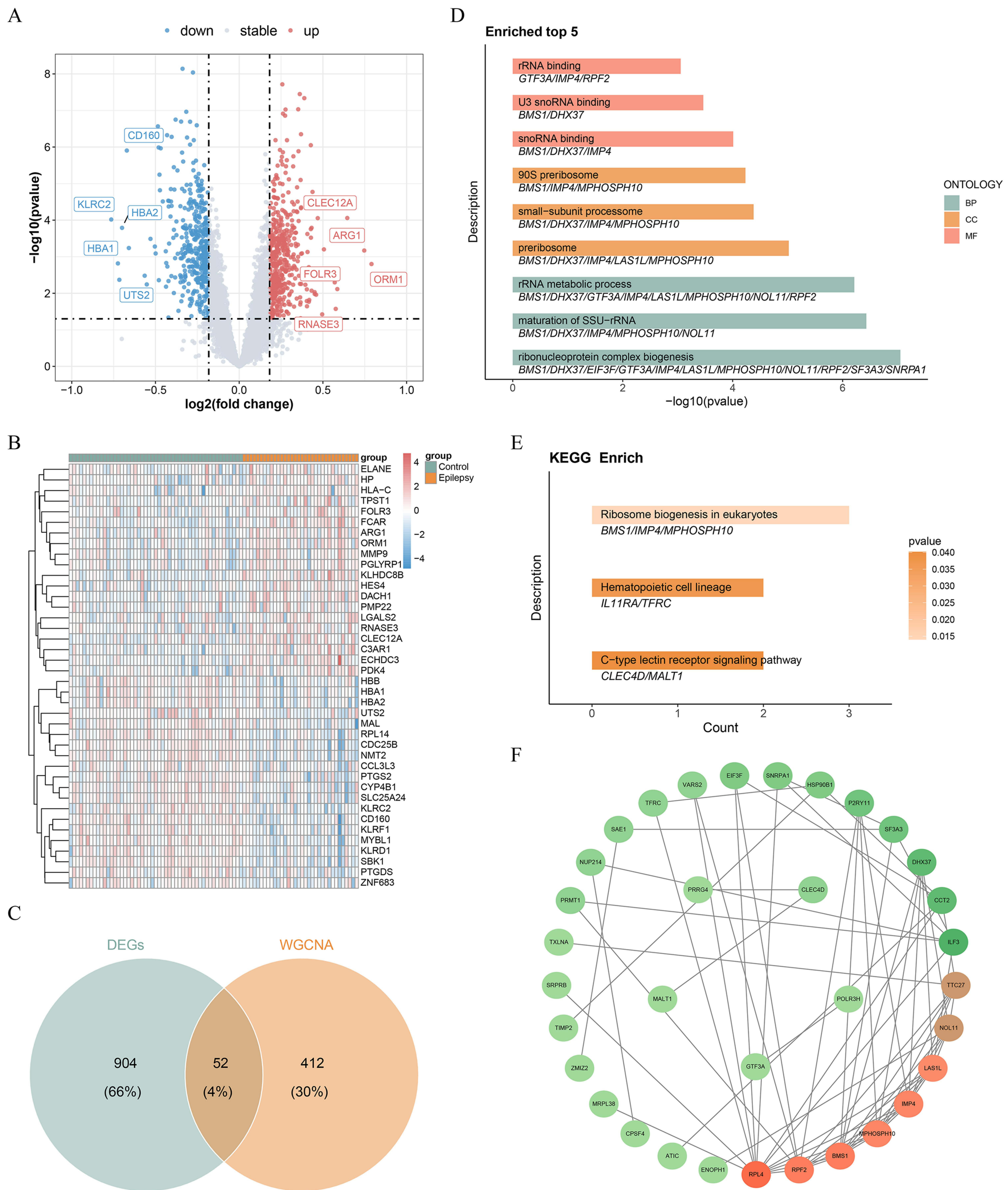

Figure 2 Identification and enrichment analysis of candidate genes. (**A**) Volcano plot showing differentially expressed genes (DEGs) between epilepsy and control groups. (**B**) Heatmap displaying the top 20 up-regulated and down-regulated genes. (**C**) Venn diagram showing the intersection between module genes and DEGs. (**D** and **E**) Functional enrichment analyses of candidate genes. (**D**) Gene Ontology (GO) enrichment analysis. (**E**) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. (**F**) Protein-protein interaction (PPI) network of candidate genes.

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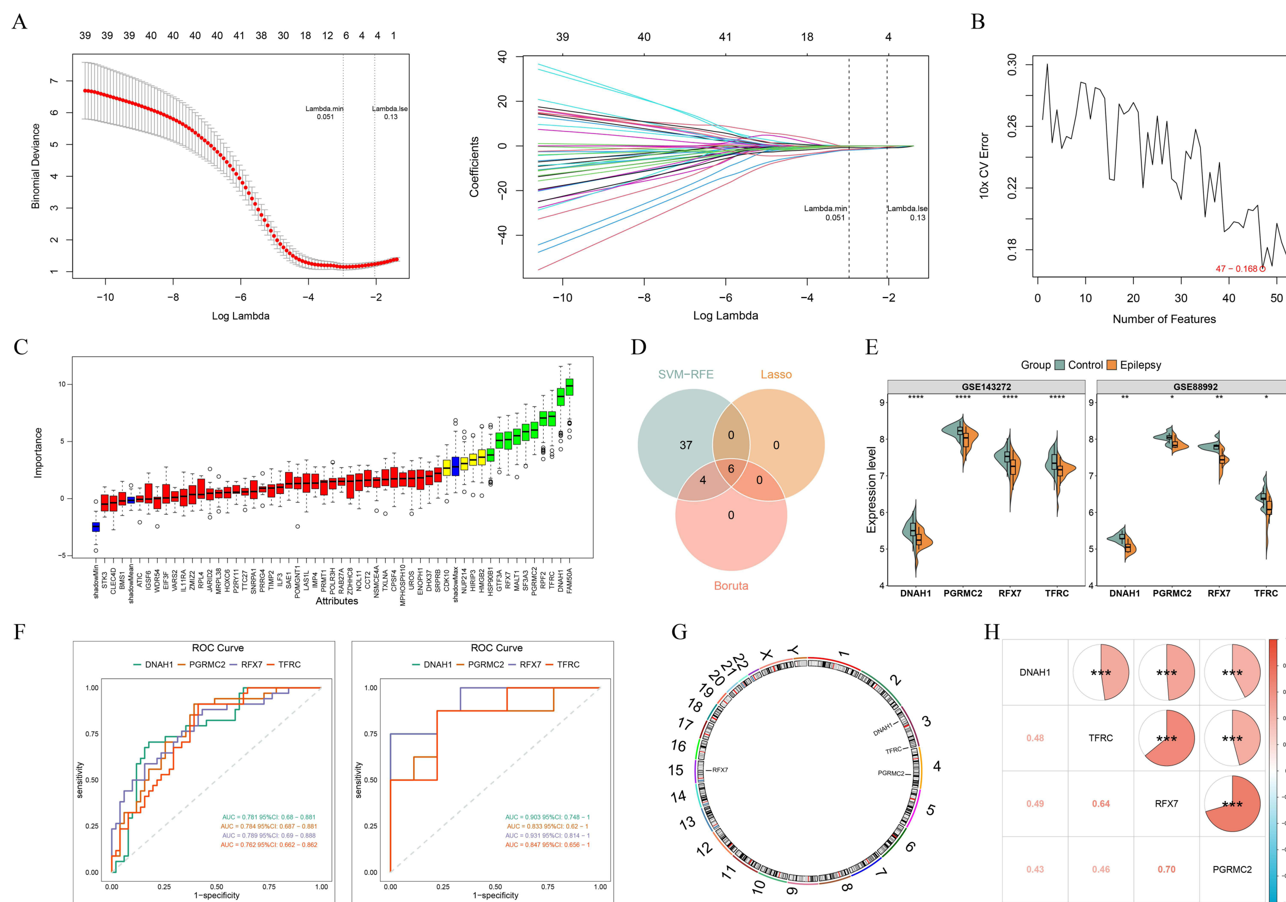

Figure 3 Identification of macrophage polarization-related biomarkers for epilepsy. (**A**) Least absolute shrinkage and selection operator (LASSO) COX regression analysis. (**B**) Support vector machine-recursive feature elimination (SVM-RFE). (**C**) Boruta feature selection. (**D**) Venn diagram showing the intersection of characteristic genes identified by three machine learning algorithms. (**E**) Expression levels of intersecting characteristic genes in independent datasets (from left to right: GSE143272 and GSE88992). (**F**) Receiver operating characteristic (ROC) curves evaluating the diagnostic performance of biomarkers in GSE143272 and GSE88992. (**G**) Chromosomal localization of identified biomarkers. (**H**) Correlation analysis among biomarkers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Functional Enrichment Analysis of Biomarkers

To elucidate the involvement of biomarkers in epilepsy, GSEA was performed. Through a comprehensive analysis of the results, we found similarities in the functions of these four biomarkers (Supplementary Table 1). Supplementary Table 1 is provided in Excel format. Specifically, all biomarkers were significantly associated with the "Complement and coagulation cascades," "Pyruvate metabolism," "Epithelial cell signaling in Helicobacter pylori infection," and "Leishmania infection" (Figure 6A–D). PGRMC2, RFX7, and TFRC were markedly associated with immune-related pathways (such as "Toll-like receptor signaling pathway," "Fc gamma R-mediated phagocytosis," "Leukocyte transendothelial migration"), genetic information processing-related pathways (such as "Ribosome," "DNA replication," "RNA degradation"), metabolism-related pathways (such as "purine metabolism," "N glycan biosynthesis," "Alanine aspartate and glutamate metabolism"), and other pathways (Figure 6B–D). DNAH1, PGRMC2, and RFX7 were considerably correlated with "Oxidative phosphorylation" and "Parkinson's disease" (Figure 6A–C). In addition, an mRNA–drug interaction network was constructed to explore potential therapeutic implications of the identified biomarkers (Figure 6G). In summary, these findings suggested that biomarkers had crucial roles in various biological pathways, highlighting their potential involvement in the pathophysiology of epilepsy.

Immune Correlation Analysis and Construction of the mRNA-Drug Network

Given the biomarkers linked to immune-related pathways, we further measured the correlations between biomarkers and 28 immune cells, as well as between the biomarkers and the ssGSEA-derived macrophage polarization score, using

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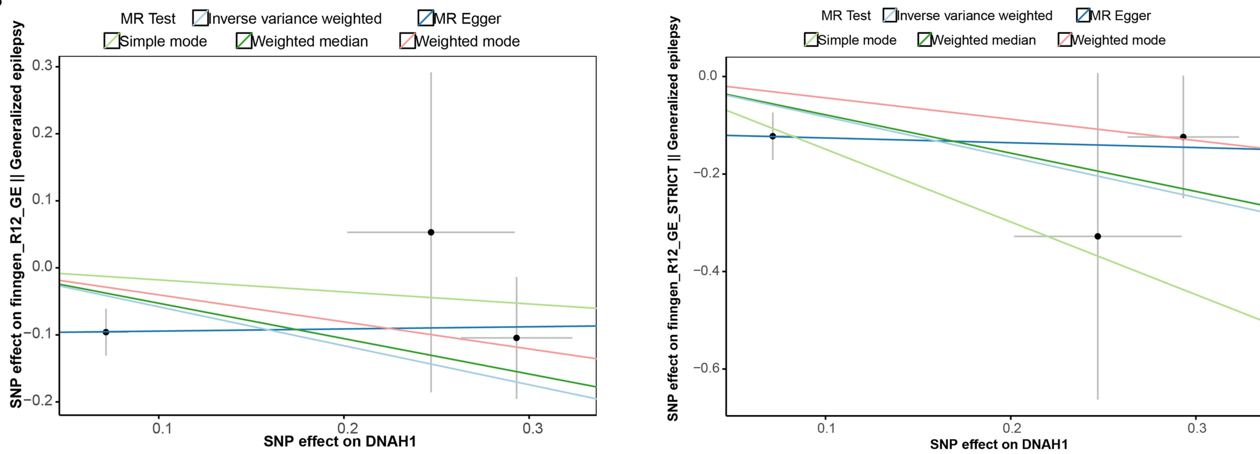
Spearman correlation ($P < 0.05$). Results revealed that among immune cells with significant differences in infiltration abundance, all biomarkers had significantly positive associations with activated CD8 T cells, activated CD4 T cells, and natural killer T cells, and had markedly negative correlations with activated dendritic cells, macrophages, mast cells, monocytes, and neutrophils (Figure 6D). The correlation between TFRC and activated CD4 T cells was the highest (cor = 0.492, P = 1.661e-06), and there was the highest negative association between RFX7 and neutrophil (cor = −0.673, P = 1.631e-12). Moreover, all biomarkers had markedly positive relevance to the MPRGs score (DNAH1: cor = 0.25, P = 0.023; PGRMC2: cor = 0.26, P = 0.019; RFX7: cor = 0.25, P = 0.019; TFRC: cor = 0.27, P = 0.014) (Figure 6E). Additionally, drug prediction indicated that a total of 64 drugs were forecasted with RFX7, TFRC, and PGRMC2 as targets. As shown in Figure 6F, the mRNA-drug network was created, including 67 nodes and 77 edges. For instance,

A

exposure	outcome	method	nsnp	pval	OR(95% CI)	pleio_P
TFRC	finngen_R12_GE || Generalized epilepsy	IVW	7	0.599	1.044 (0.890 to 1.225)	0.793
DNAH1	finngen_R12_GE || Generalized epilepsy	IVW	3	0.021	0.559 (0.341 to 0.918)	0.323
TFRC	finngen_R12_GE_STRICT || Generalized epilepsy	IVW	7	0.296	1.126 (0.902 to 1.406)	0.776
DNAH1	finngen_R12_GE_STRICT || Generalized epilepsy	IVW	3	0.018	0.438 (0.220 to 0.870)	0.366

0 0.5 1 1.5 2

B



C

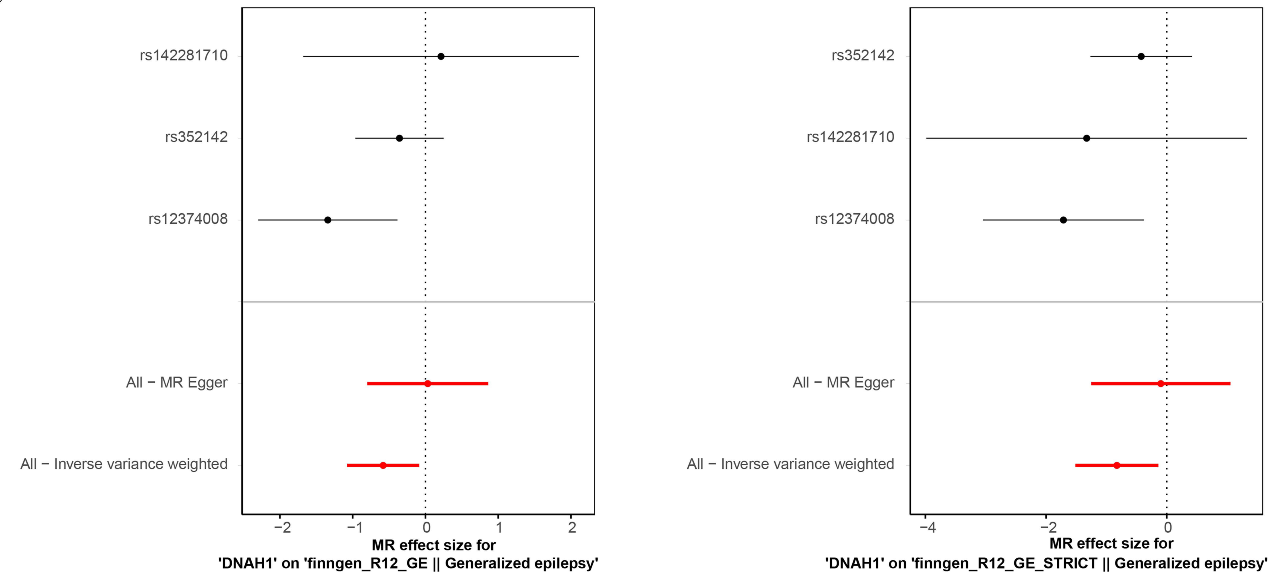
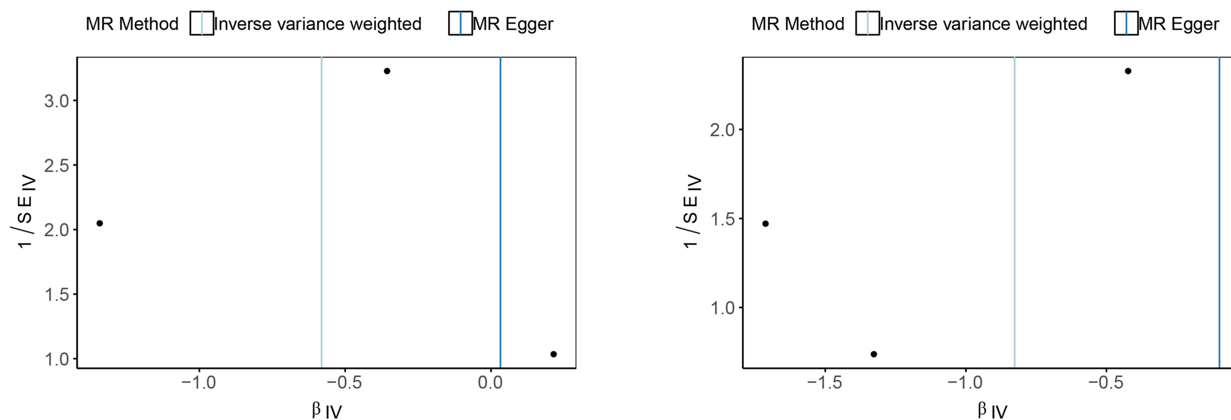


Figure 4 continued.



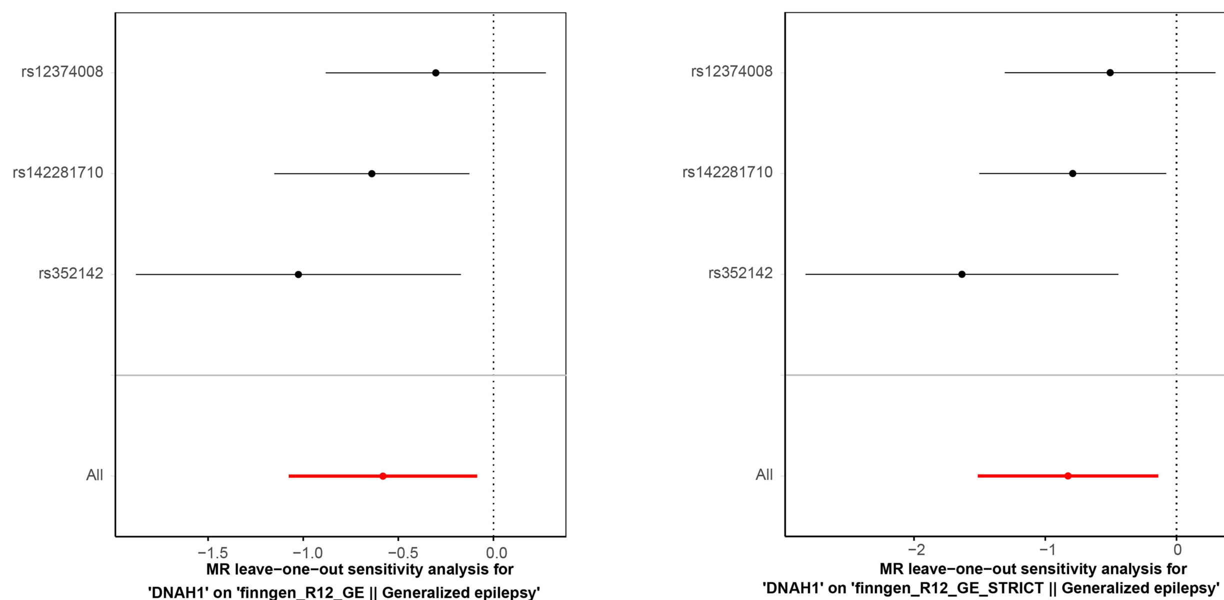


Figure 4 Causal effects of biomarkers on epilepsy based on Mendelian randomization (MR) analysis. (**A**) Forest plot showing the causal effects of biomarkers on epilepsy. (**B**) Scatter plots illustrating the causal effect estimates of DNAH1 on epilepsy using two FinnGen datasets (from left to right: finngen_R12_GE and finngen_R12_GE_STRICT). (**C**) Forest plots of causal estimates for DNAH1. (**D**) Funnel plots assessing heterogeneity and potential pleiotropy. (**E**) Leave-one-out (LOO) sensitivity analysis of DNAH1. Red text in the p-value column indicates statistically significant results ($P < 0.05$).

estradiol had interactions with three targets. The bucladesine, cephaeline, coumestrol, doxorubicin, sanguinarine, staurosporine, and verteporfin all interacted with TFRC and PGRMC2. Additionally, camptothecin, daunorubicin, doxorubicin, and tyrphostin AG-825 interacted with RFX7 and PGRMC2.

Cellular Distribution of Biomarkers

To evaluate the cellular distribution of the biomarkers, single-cell analysis was conducted. A total of 26 clusters were identified and annotated into 8 cell types: microglia, T cells, neurovascular unit (NVU) cells, macrophages, oligodendrocytes, B cells, natural killer (NK) cells, and astrocytes (Figure 7A–C and Supplementary Figure 1). In epilepsy samples, microglia accounted for the largest proportion among all cell types (Figure 7D). All biomarkers were detected across multiple cell types, with DNAH1 and PGRMC2 predominantly detected in astrocytes, RFX7 in T cells, and TFRC in microglia (Figure 7E). Additionally, cell-cell interaction analysis illustrated communication patterns among these cell types in the epilepsy microenvironment (Figure 7F). The results demonstrated active intercellular communication, with the strongest interaction observed between microglia and macrophages. It is worth noting that the single-cell dataset used

Table 2 Heterogeneity and Pleiotropy Analyses of Mendelian Randomization Results

Outcome	Exposure	MR Method	Q	Q_df	Q_pval	Egger Intercept	SE	P-value
Epilepsy (finngen_R12_GE)	DNAH1 (eqtl-a-ENSG00000114841)	MR Egger	0.390	1	0.533	−0.098	0.054	0.323
		Inverse variance weighted	3.632	2	0.163	—	—	—
Epilepsy (finngen_R12_GE_STRICT)	DNAH1 (eqtl-a-ENSG00000114841)	MR Egger	0.341	1	0.559	−0.116	0.075	0.366
		Inverse variance weighted	2.715	2	0.257	—	—	—

Notes: Egger intercept and corresponding P-values indicate horizontal pleiotropy. "—" indicates not applicable.
Abbreviations: MR, Mendelian randomization; Q, Cochran's Q statistic for heterogeneity; Q_df, degrees of freedom; SE, standard error.

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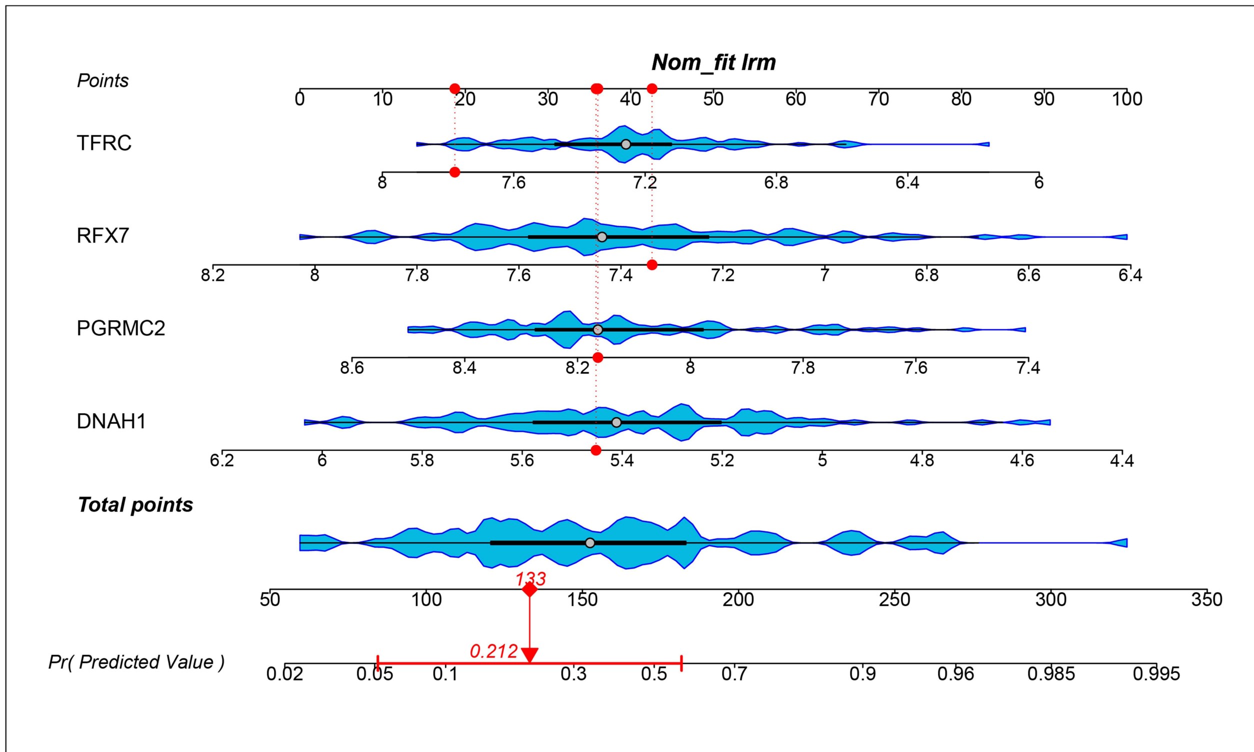

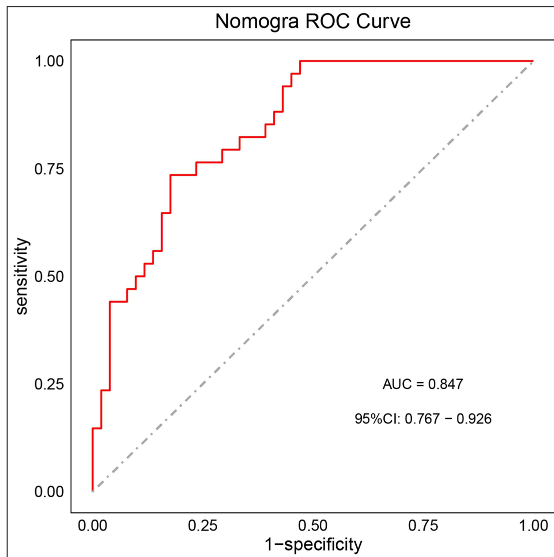

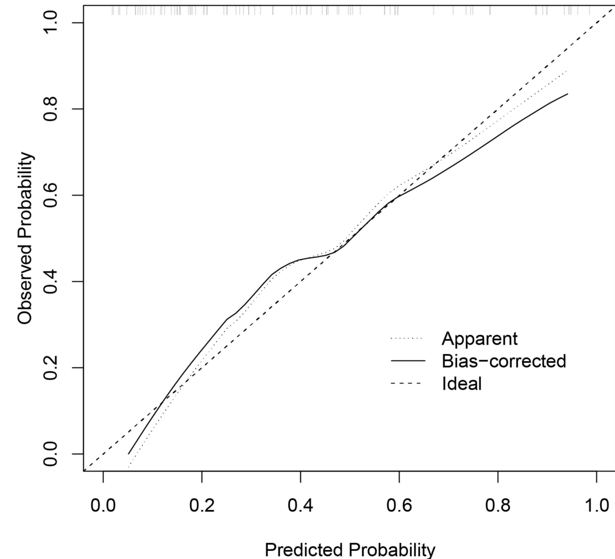

Figure 5 Construction and evaluation of the diagnostic nomogram. (**A**) Nomogram integrating identified biomarkers. (**B**) Receiver operating characteristic (ROC) curves evaluating diagnostic performance. (**C**) Calibration curves assessing the agreement between predicted and observed outcomes.

in this study included only epilepsy samples without healthy controls; therefore, the single-cell analysis was performed to characterize the cellular distribution of the biomarkers rather than to assess differential expression at the single-cell level.

Validation of the Expression of Biomarkers in Blood Samples

To determine whether the bioinformatically identified candidate biomarkers could be validated in peripheral blood samples, RT-qPCR and Western blot analyses were performed. Accordingly, peripheral blood samples were collected

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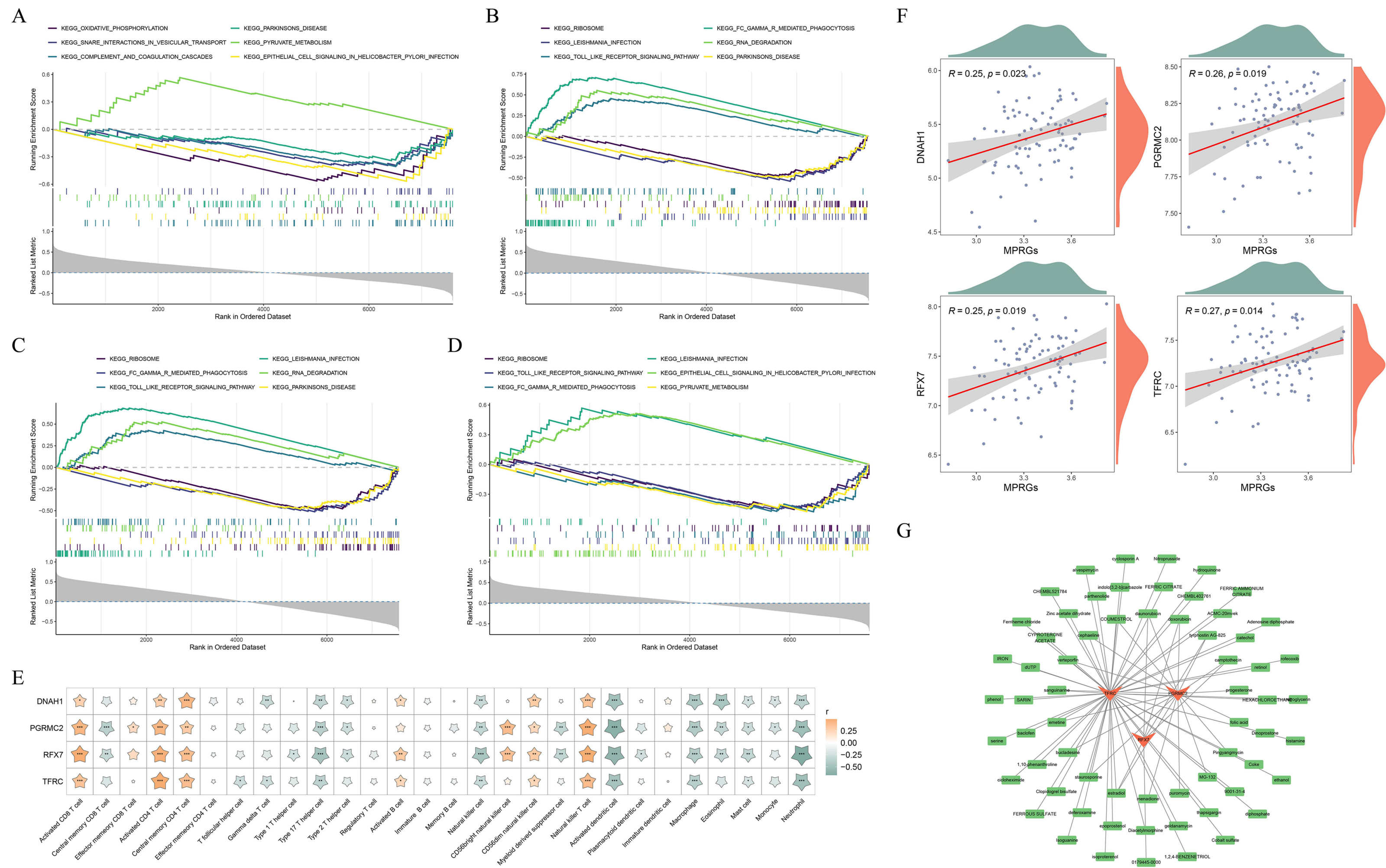

Figure 6 Exploration of potential mechanisms by which biomarkers modulate epilepsy. (**A**–**D**) Gene set enrichment analysis (GSEA) of biomarkers. (**A**) DNAH1. (**B**) PGRMC2. (**C**) RFX7. (**D**) TFRC. (**E**) Correlation analysis between biomarkers and 28 immune cell types. (**F**) Associations between biomarkers and macrophage polarization-related gene (MPRG) scores. (**G**) mRNA–drug interaction network. Red nodes represent mRNAs and green nodes represent drugs. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

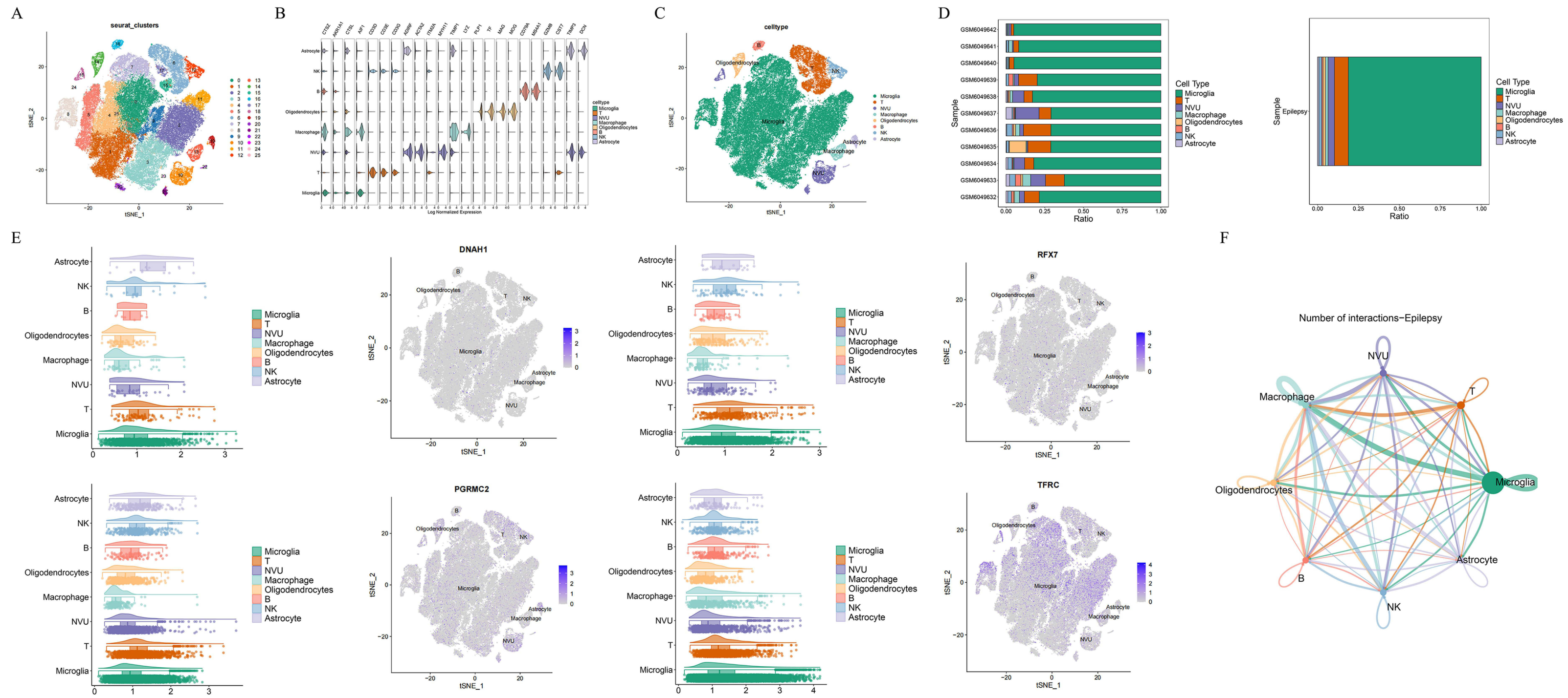

Figure 7 Single-cell transcriptomic analysis. (**A**) t-distributed stochastic neighbor embedding (t-SNE) plot showing 26 independent clusters. (**B**) Expression of marker genes in annotated cell types. (**C**) t-SNE plot showing 8 major cell types. (**D**) Proportions of each cell type. (**E**) Expression levels of biomarkers across different cell types (from top to bottom: DNAH1, PGRMC2, RFX7, and TFRC). (**F**) Cell–cell communication network illustrating the number and strength of interactions among cell types.

from epilepsy patients and healthy controls for experimental validation. RT-qPCR results indicated that, compared with the control group, the expression levels of DNAH1 and TFRC were markedly decreased in the epilepsy group (Figure 8A). However, no statistically significant differences in the expression of PGRMC2 and RFX7 were observed between the epilepsy and control groups (Figure 8A). Because a reliable antibody for RFX7 was unavailable, the protein expression of the remaining three biomarkers was examined. The results showed that the protein expression levels of DNAH1, PGRMC2, and TFRC were all lower in the epilepsy group than in the control group (Figure 8B). Collectively, only DNAH1 and TFRC showed consistent changes at both the transcriptional and protein levels that agreed with the bioinformatics analysis.

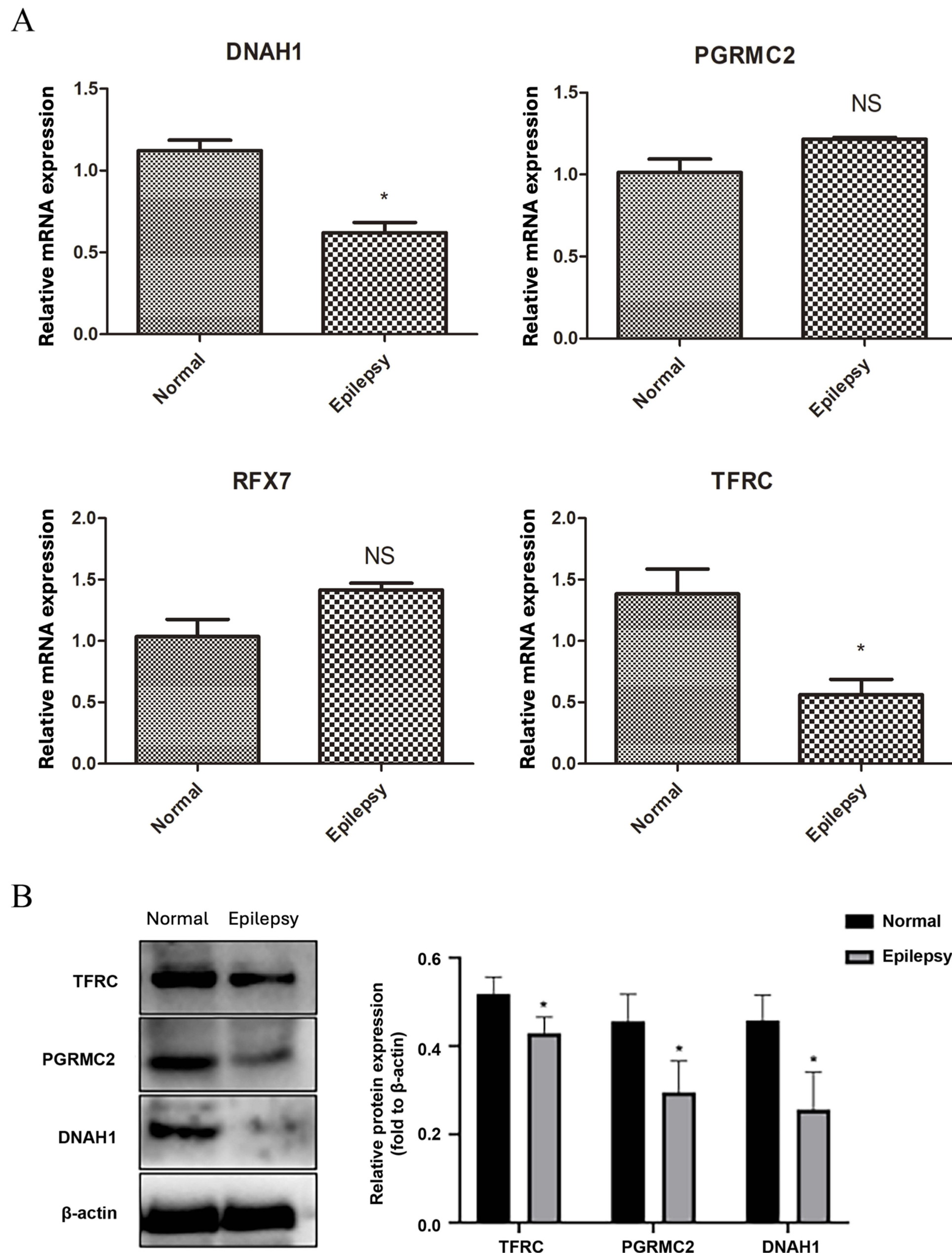

Figure 8 Validation of biomarker expression at transcriptional (mRNA) and protein levels. (**A**) Relative mRNA expression levels of biomarkers (from left to right: DNAH1, PGRMC2, RFX7, and TFRC). (**B**) Protein expression levels of biomarkers determined by Western blot analysis. *P < 0.05.
Abbreviation: ns, not significant.

Discussion

The pathophysiological process of epilepsy is closely intertwined with neuroinflammation, in which brain macrophages, particularly microglia, play a central role.[20] Depending on local microenvironmental cues, microglia can polarize toward a pro-inflammatory M1 phenotype or an anti-inflammatory and reparative M2 phenotype, a phenomenon observed in multiple neurodegenerative disorders.[21] Notably, during seizures and in chronic epileptic states, microglia rapidly adopt the M1 phenotype, releasing pro-inflammatory mediators such as TNF-α and IL-1β. This response amplifies inflammation, lowers the seizure threshold,[22] disrupts the blood–brain barrier, and exacerbates neuronal injury.[23] Bröer et al[22] demonstrated that M1-type microglia in the brains of epilepsy patients highly express pro-inflammatory genes such as IL1B and CCL4, confirming their involvement in human epilepsy. Conversely, driving microglia toward the M2 phenotype can suppress inflammation and promote tissue repair, thereby exerting an antiepileptic effect and offering potential targets for novel therapeutic strategies.[24,25] In our study, we identified four biomarkers associated with macrophage polarization—DNAH1, PGRMC2, RFX7, and TFRC. These biomarkers may provide new entry points for dissecting the neuroimmune mechanisms of epilepsy and potential targets for developing immune-modulatory therapies.

DNAH1 (Dynein Axonemal Heavy Chain 1) encodes a heavy-chain subunit of the inner dynein arm of axonemal microtubules. It is typically expressed in motile ciliated or flagellated cells, most prominently in sperm flagella and multiciliated epithelia such as those of the respiratory tract, fallopian tubes, and ventricular ependyma.[26] Although no direct evidence links DNAH1 to macrophage (M1/M2) or microglial polarization—or to epilepsy itself—its expression in ependymal cells suggests potential relevance to central nervous system (CNS) function. The rhythmic beating of ependymal cilia is essential for maintaining normal cerebrospinal-fluid (CSF) circulation; disruption of this process may impair brain microenvironmental homeostasis.[27] We speculate that aberrant DNAH1 expression may alter CSF dynamics and consequently affect the cerebral immune milieu—such as cytokine distribution—thereby contributing indirectly to neuroinflammation and epileptogenesis. Although the precise mechanisms linking peripheral DNAH1 expression to epilepsy remain speculative, alterations in blood–brain barrier integrity and systemic immune signaling may represent potential connecting pathways.

PGRMC2 (Progesterone Receptor Membrane Component 2) is a membrane-associated protein broadly expressed in neurons, astrocytes, and microglia. Localized mainly to the endoplasmic reticulum and nuclear envelope,[28] it functions as a heme-binding chaperone that mediates intracellular heme transport to the nucleus, thereby influencing gene expression and mitochondrial activity.[29,30] Under pathological conditions, PGRMC2 appears to regulate both neuroinflammatory and epileptic processes. In a middle cerebral artery occlusion model, Zhou et al[31] reported that microglial PGRMC2 expression remained largely unchanged after ischemic stroke; however, its activation reduced M1 microglial markers (Iba-1, CD68) and increased M2 markers (CD206). In epilepsy models, PGRMC2 expression in the hippocampus was down-regulated, leading to enhanced neuronal excitability and aggravated seizure activity.[28] Likewise, ketogenic diet–induced up-regulation of PGRMC2 has been shown to preserve mitochondrial integrity and reduce neuronal death, conferring an antiepileptic effect.[32] Consistent with these reports, both bioinformatic and experimental analyses in our study demonstrated a marked down-regulation of PGRMC2 in epilepsy, reinforcing its role as a key molecular component in the disease's pathophysiological network.

RFX7 (Regulatory Factor X7), a member of the RFX transcription-factor family, is localized to the nucleus and highly expressed in various brain regions, including both inhibitory and excitatory cortical neurons.[33] Single-cell datasets indicate that RFX7 can also be detected in CNS macrophage-like cell populations. Functionally, RFX7 is activated by p53 and directly regulates tumor-suppressive or growth-inhibitory genes such as PDCD4 and PIK3IP1, integrating signals that control cell growth and fate determination.[34] RFX7 haploinsufficiency has been linked to several CNS disorders, notably autosomal dominant intellectual disability type 71 (MRD71), characterized by global developmental delay, cognitive and language impairment, autism-spectrum disorder (ASD), and attention-deficit/hyperactivity disorder (ADHD). Epileptic seizures have been reported in a subset—but not all—of these patients.[35] Altogether, RFX7 emerges as a pivotal transcription factor in CNS development and function, and its dysregulation may contribute to complex neurodevelopmental and neuroimmune phenotypes, suggesting an underexplored role in epileptogenesis.

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TFRC encodes transferrin receptor 1 (TfR1 or CD71), a widely expressed type II transmembrane glycoprotein abundantly present in erythroid cells, the nervous system, placenta, and proliferating tissues. Its primary function is to mediate cellular uptake of iron bound to transferrin, thereby maintaining iron homeostasis.[36–38] In CNS disorders, TFRC influences disease progression mainly through iron-metabolism dysregulation and ferroptosis. Iron overload contributes to neurodegenerative diseases such as Alzheimer's and Parkinson's, and aberrant TFRC expression has been implicated in brain iron accumulation.[39,40] In stroke or CNS injury, TFRC is up-regulated, promoting ferroptosis, neuronal death, and inflammation.[41] During epileptogenesis, however, TFRC expression is markedly down-regulated and correlates strongly with the immune microenvironment of epilepsy.[42] The ketogenic diet (KD) has been shown to down-regulate TFRC via HDAC4-mediated deacetylation, protecting neurons from ferroptotic injury in epilepsy.[43] Moreover, TFRC has been identified as a hub gene in epilepsy and is co-regulated with other ferroptosis-related genes such as RELA and QSOX1.[44,45] In our data, TFRC was significantly reduced in epilepsy compared with controls. Given its close association with the immune microenvironment, we propose that TFRC down-regulation not only disrupts neuronal iron homeostasis directly but may also reshape immune–inflammatory signaling, jointly driving the development and progression of epilepsy.

Both the GSEA and immune infiltration analyses revealed the pivotal roles of the macrophage polarization–related biomarkers—DNAH1, PGRMC2, RFX7, and TFRC—in modulating immune-inflammatory responses in epilepsy. All four markers showed negative enrichment in the complement and coagulation cascades pathway, suggesting that their downregulation might help suppress excessive complement activation and, in turn, mitigate the amplification of complement-driven neuroinflammation.[46] It should be noted that the Mendelian randomization analysis in this study was based on blood-derived eQTL data, whereas epilepsy primarily affects the brain. Blood eQTLs may not fully capture gene regulatory mechanisms in brain tissues relevant to epilepsy. Therefore, the inferred causal association, particularly for DNAH1, should be interpreted with caution. The complement system promotes cytokine release and microglial activation, which together enhance neuronal hyperexcitability and seizure activity.[47] In experimental models of epilepsy, aberrant complement components such as C3 contribute to abnormal synaptic pruning and neurodegeneration, thereby creating a pro-epileptogenic environment.[48] Immune infiltration analysis further indicated that the expression of these biomarkers was negatively correlated with macrophage infiltration in epileptic samples. This implies that low expression levels may facilitate macrophage recruitment and/or promote M1-type pro-inflammatory polarization through certain mechanisms.[22] Macrophages, including microglia and infiltrating monocytes, exhibit a dual role: the M1 phenotype releases neurotoxic cytokines such as TNF-α and IL-1β, whereas the M2 phenotype promotes inflammation resolution.[49] A persistent M1-dominant state in epileptic brain regions has been linked to recurrent seizures; for example, IL-6 can exacerbate seizure activity in temporal lobe epilepsy models. Based on these findings, we speculate that the identified biomarkers may exert their effects by attenuating complement activation, thereby limiting M1 activation and promoting a shift toward the M2 phenotype. This mechanism could help disrupt the vicious inflammatory cycle—similar to how MyD88 inhibition has been shown to alter polarization states and reduce apoptosis after status epilepticus.[50] However, the precise pathways involved remain to be validated through further experimental and clinical studies.

In summary, by integrating multilayer bioinformatic analyses with experimental validation, this study identified DNAH1, PGRMC2, RFX7, and TFRC as macrophage polarization–related candidate biomarkers associated with epilepsy. These molecules appear to participate in the regulation of immune-inflammatory processes, such as complement and coagulation cascades, as well as metabolic pathways. Importantly, validation using clinical blood samples further supported the consistent expression patterns of DNAH1 and TFRC. This integrative strategy—from data mining and mechanistic inference to experimental verification—contributes to a deeper understanding of immune regulation in epilepsy and highlights potential targets for macrophage phenotype–based therapeutic strategies.

Of note, although PGRMC2 and RFX7 were identified as candidate biomarkers through integrated bioinformatics analyses, they did not show statistically significant differences in peripheral blood samples. This inconsistency may be attributed to several factors. First, the relatively small sample size of the clinical validation cohort (n = 7 epilepsy patients vs. n = 6 controls) may have limited the statistical power to detect modest expression differences. Second, the bioinformatics analyses were primarily based on brain-derived transcriptomic datasets, whereas the experimental validation was performed using peripheral blood samples. Tissue-specific gene regulation may therefore contribute to

the observed discrepancy. Future studies with larger cohorts and brain-derived samples are warranted to further clarify the clinical relevance of PGRMC2 and RFX7.

Nevertheless, several limitations should be acknowledged. First, the sample sizes of the datasets used in this study were relatively small (eg, the training set GSE143272 included 85 cases, the validation set GSE88992 consisted of 17 mouse samples, and the single-cell dataset contained 11 epileptic brain tissues), which may introduce bias. In addition, cross-species differences between mouse models and human epilepsy warrant further consideration. Second, the macrophage polarization–related gene set used in this study was originally derived from a COPD-related transcriptomic analysis. Although curated to reflect macrophage polarization states rather than disease-specific mechanisms, and given that macrophage polarization represents a conserved immune program across inflammatory conditions, its applicability to epilepsy may be limited. Therefore, epilepsy-specific macrophage polarization signatures warrant further refinement in future studies. Third, the mechanistic exploration in this study was largely based on correlation analyses, as functional experiments such as gene knockout or overexpression were not performed to directly validate the roles of these biomarkers in regulating macrophage polarization or seizure activity. Fourth, the clinical validation results showed no significant mRNA-level changes for PGRMC2 and RFX7, and RFX7 could not be validated at the protein level due to the lack of a reliable antibody, limiting the comprehensiveness of the experimental validation. Moreover, potential confounding factors such as age, sex, and epilepsy subtype were not systematically evaluated. Therefore, further in vivo and in vitro studies are required to substantiate these findings and clarify the precise roles of these biomarkers in the pathogenesis of epilepsy.

Conclusion

Through multi-algorithm bioinformatic analyses, this study identified four macrophage polarization–related candidate biomarkers associated with epilepsy—DNAH1, PGRMC2, RFX7, and TFRC—with robust discriminatory performance across independent cohorts. Functional evidence indicates that these genes are involved in immune-inflammatory processes, particularly the complement–coagulation pathway, potentially linking glial polarization and neurovascular unit remodeling in epileptogenesis. Notably, DNAH1 and TFRC were further supported by clinical blood-based validation, underscoring their translational relevance in epilepsy.

Data Sharing Statement

All datasets analyzed in this study are publicly available in the Gene Expression Omnibus (GEO) database under accession numbers GSE143272, GSE88992, and GSE201048 (https://www.ncbi.nlm.nih.gov/geo/). Macrophage polarization–related gene sets were obtained from the MSigDB database (https://www.gsea-msigdb.org/gsea/msigdb). Additional data generated during this study are available from the corresponding author upon reasonable request.

Ethics Statement

This study was approved by the Ethics Committee of the First Hospital of Shanxi Medical University (KYLL-2025-066). Written informed consent was obtained from all participants or their legal guardians in accordance with the Declaration of Helsinki. This study was conducted and reported in accordance with the RECORD (REporting of studies Conducted using Observational Routinely-collected Data) reporting guidelines.

Consent for Publication

Not applicable. No identifiable personal information or images were included in this study.

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Author Contributions

Jiyuan Li: Conceptualization; Methodology; Validation; Statistical analysis; Writing – original draft; Supervision.

Chenlu Zhang: Conceptualization; Bioinformatics analysis; Statistical analysis; Writing – original draft.

Hui Zhang: Methodology; Bioinformatics analysis; Writing – review & editing.

Xuefeng Wang: Methodology; Writing – review & editing; Supervision.

Xiaoping Yang: Validation; Clinical sample collection; Writing – review & editing.

Yihan Liu: Statistical analysis; Data curation; Writing – review & editing.

Zhijuan Zhang: Statistical analysis; Data curation; Writing – review & editing.

All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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