



OPEN Uncovering the role of integrated stress in Alzheimer's disease through single-cell and transcriptomic analysis

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Alzheimer's disease (AD) is a common neurodegenerative disorder; however, its molecular complexity remains poorly understood. Single-cell analysis can reveal the molecular changes in AD in different types of brain cells. In this study, we integrated single-cell sequencing and transcriptome data to explore the molecular mechanism of integrated stress response (ISR) in AD. Analysis of the GSE264648 (49 cases) and GSE48350 (253 cases) datasets showed that the integrated stress response (ISR) activity of endothelial cells in patients with AD was significantly increased compared with normal control group. Six key genes (*BTG1*, *EPB41L4A*, *HERPUD1*, *SLC3A2*, *SLC7A11*, and *SLC7A5*) were screened by combining the Least Absolute Shrinkage and Selection Operator (LASSO) regression and the random forest algorithm. Urine test for β -amyloid protein, Clinical Dementia Rating, modified Hachinski Ischemia Scale, Hamilton Depression Scale, Hamilton Anxiety Scale and head magnetic resonance imaging were used to screen clinical subjects, and then verified the six key genes in their blood samples. These key genes are enriched in inflammatory pathways such as NF- κ B and TNF, and are closely related to immune cell infiltration (e.g., M2 macrophages and neutrophils). This research also revealed the association between key and core genes of AD (e.g., APOE) and their clinical predictive value, providing new clues for mechanistic research and targeted therapy of AD.

Keywords Alzheimer's disease, Integrated stress response, Single-cell sequencing, Key genes, Clinical validation

Alzheimer's disease (AD) is a global health concern that causes loss of neuropsychological function and cognitive ability. The prevalence of AD has been increasing annually with the advent of an aging society. Among the ten most common diseases that cause death worldwide, it is a disease that lacks effective prevention and treatment programs. It is of great scientific value and practical significance to study the pathogenesis of AD and explore effective prevention and treatment methods. The pathology of AD mainly involves the formation of amyloid plaques and neurofibrillary tangles, leading to neuronal damage and death¹. Abnormal metabolism of amyloid precursors produces beta-amyloid proteins, which accumulate in the brain, while abnormal phosphorylation of tau protein results in the formation of tangles, affecting the function of nerve cells. In terms of epidemiology, AD is the most common type of dementia, with approximately 50 million patients worldwide, and it is expected to increase to 152 million by 2050. The incidence of AD increases with age, and approximately 10% of people over 65 years of age suffer from the disease. The incidence rate in women is higher than that in men, and both genetic and environmental factors are thought to affect the risk of the disease².

Treatments for AD mainly includes drug therapy and non-drug interventions. So far, the drugs approved by the U.S. Food and Drug Administration for the treatment of AD mainly include acetylcholinesterase inhibitors, n-methyl-D-aspartate receptor antagonists, and anti-beta-amyloid (β -amyloid, A β) monoclonal antibodies³.

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However, these drugs can only improve some of the clinical symptoms of AD, and it is crucial to mine more potential key genes to explore the mechanism of AD and develop AD management drugs. Non-drug interventions involve cognitive training and social activities to improve quality of life. In prognostic management, regular monitoring of cognitive and daily living abilities is essential, and family support and education are also important to help provide better care for patients. The overall goal is to delay the course of the disease and improve functional status and quality of life⁴.

Bioinformatics is an emerging discipline widely used in various biomedical research fields⁵. Bioinformatics involves the integrated analysis of multiple databases using a computer⁶, which provides a relatively quick and convenient research method for exploring the pathogenesis and potential targets of diseases, with particular significance for the diagnosis and treatment of patients. With continuous progress in science and technology, single-cell RNA transcriptome (scRNA-seq) technology has become increasingly mature and has been applied to embryo, tumor, stem cell, nervous system, and other fields^{7,8}. scRNA-seq can detect differences in cellular gene transcription levels at the single-cell level, which is conducive to the discovery of new cells. Differences between tissues and cells can be determined by accurately measuring the levels of intracellular gene expression, providing a basis for the precise treatment of AD. In early embryonic research, this technology was used to track the developmental processes of embryonic cells and reveal their developmental trajectory. This technique can also be used to study cell-cell interactions and identify co-regulatory gene modules⁹. In terms of data processing, how to more accurately describe the biological function of cells at the molecular level and how to accurately determine cell types and define cell subsets through transcriptome data requires greater room for progress¹⁰. The application of scRNA-seq technology provides a deeper understanding of the pathophysiological mechanism of diseases and provides new ideas and targets for the diagnosis and treatment of diseases. Microarray gene chip technology has been used to hybridize DNA samples with gene chips covered with probes to detect chromosomal abnormalities at the whole-genome level. Gene chips can not only detect chromosome aneuploidy abnormalities, but can also detect chromosome copy number variations and heterozygosity loss over 50–100 kb^{11,12}, which is of great significance for the early diagnosis of neurodegenerative diseases.

Materials and methods

Bioinformatics analysis

Data acquisition

Data from 49 GSE264648 samples (25 AD samples and 24 control samples) were downloaded from the NCBI GEO public database (<https://www.ncbi.nlm.nih.gov/geo/info/datasets.html>) for single-cell analyses¹³. The Series Matrix file of GSE48350 was downloaded from the NCBI GEO public database, and the annotation file GPL570 included the expression profile data of 253 patients, with 173 in the control group and 80 in the AD group.

Single-cell analysis

The expression profile was read in through the Seurat package, in which we filtered the cells according to the total number of unique molecular identifiers (UMIs) in each cell, the number of expressed genes, and the mitochondrial and ribosome expression ratios of each cell¹⁴. The median absolute deviation (MAD) was used for quality control¹⁵. Data with more than three times the MAD were generally considered outliers. DoubletFinder (V2.0.4) was used to filter the double cells of each sample separately, thereby completing cell quality control.

Data standardization

We used the global normalization LogNormalize method to adjust the total expression of each cell to 10,000 by multiplying a coefficient s_0 , and then took the logarithm for standardization¹⁶. CellCycleScoring was used to calculate the cell cycle score, and FindVariableFeatures was used to find highly variable genes¹⁷. The ScaleData function was used to remove gene expression fluctuations caused by the expression ratio of mitochondrial genes, the expression ratio of ribosomal genes, and the different cycles of cells¹⁸. RunPCA was employed to perform linear dimensionality reduction on the expression matrix and select the principal components for subsequent analysis¹⁹. Harmony was used to remove batch effects, and RunUMAP unified manifold approximation and projection (UMAP) was used for nonlinear dimensionality reduction²⁰. The clusters were annotated using the CellMarker database, and cells that were important to the disease were annotated²¹. SingleR software automated annotation was used to identify the cell types and corresponding marker genes in the corresponding tissues for cell annotation²².

Ligand-receptor interaction analysis (CellChat)

In this analysis, we used the standardized single-cell expression profile as input data and the cell subtypes obtained from the single-cell analysis as cell information²³. We analyzed cell-related interactions and quantified the closeness of the interaction relationship using the interaction strength (weights) and count between cells to observe the activity and influence of each cell type on the disease¹⁴.

Least absolute shrinkage and selection operator (LASSO) regression and random forest feature selection

LASSO logistic regression and random forest algorithms were used to select features for the diagnostic markers of the diseases. The LASSO algorithm uses the “glmnet” package¹⁴. We evaluated the importance of features using the random forest algorithm and %IncMSE, and selected the top ten features and LASSO feature genes for subsequent analysis.

Gene set enrichment analysis (GSEA)

Patients were divided into the high-expression group and the low-expression group based on the median expression values of key genes, and the differences in signaling pathways between the two groups were further analyzed using GSEA¹⁴. The background gene set was the annotation gene set downloaded from the MsigDB database as the annotation gene set of the subtype pathway, and differential expression analysis of the pathways between subtypes was performed. The significantly enriched gene set (adjusted $p < 0.05$) was ranked according to the consistency score.

Gene set variation analysis (GSVA)

In this study, we downloaded gene sets from the Molecular Signatures database and used the GSVA algorithm to comprehensively score each gene set to evaluate the differences²⁴.

Immune infiltration analysis

The CIBERSORT algorithm was used to analyze patient data to infer the relative proportions of 22 immune-infiltrating cells and to perform correlation analysis of gene expression and immune cell content²⁵.

Relevant clinical importance score

AlzData (<http://www.alzdata.org/>) is a resource website focusing on AD and other cognitive disorders, which covers the following: (1) high-throughput omics data, such as genomics (GWAS and whole exome sequencing), transcriptomics, proteomics, and functional genomics; and (2) high-confidence functional data, such as neuroimaging screening, population-based longitudinal studies, and transgenic mouse phenotyping²⁶.

Nomogram model construction

By constructing a multivariate regression model, each value level of each influencing factor was scored according to the contribution of each influencing factor in the model to the outcome variable (size of the regression coefficient)²⁷. The scores were then added together to obtain the total score, thereby calculating the predicted value.

Developmental trajectory of key cell subtypes

Monocle introduces a strategy for sorting single cells in pseudotime (pseudo-sequential) using the asynchronous process of single cells to place them on trajectories corresponding to biological processes such as cell differentiation²⁷.

Clinical validation

Diagnostic criteria

The diagnosis of AD was based on the AD Core Criteria (National Institute on Aging-Alzheimer's Association [NIA-AA], 2011)²⁸: (1) latent onset with a clear history of progressive deterioration of cognitive function; (2) history and examination confirming one of the earliest and most significant categories of cognitive impairment (amnesic and non-amnesic symptoms); and (3) ruling out other causes. Additionally, it was necessary for the patient's urine test to be positive for A β amyloid protein.

The urine β -amyloid protein detection kit (colloidal Gold lateral immunochromatography method) is produced by Hunan Gankang Technology Co., Ltd. (batch number: A20241013, license number: Xiang Food and Drug Administration Medical Device Production License No. 20160047, Registration Certificate Number). The commercial test kits of Xiangjiang Medical Device Registration (20202401261) strictly follow the procedures specified in the kit manual during the testing process. This kit achieves qualitative detection by specifically binding colloidal gold-labeled anti-A β monoclonal antibody to β -amyloid protein in urine samples, forming a visible color band on the nitrocellulose membrane²⁹.

Inclusion criteria

The inclusion criteria were as follows: (1) age ≥ 50 and ≤ 80 years; (2) met the NIA-AA 2011 diagnostic criteria for AD and the urine test for β -amyloid protein was positive; (3) The Clinical Dementia Rating (CDR) score is greater than 0 and less than 2; (4) modified Hachinski Ischemia Scale (m-HIS) total score < 4 ; (5) Hamilton Depression Scale (HAMD) (24 items) < 8 , Hamilton Anxiety Scale (HAMA) < 7 ; (6) head magnetic resonance imaging (MRI) showing hippocampal atrophy consistent with AD; (7) no obvious positive signs on nervous system examination; (8) availability of a stable and reliable caregiver to accompany the patient and assist the researchers with completing the HAMD and HAMA; and (9) provision of written informed consent by the patients and their families.

Exclusion criteria

The exclusion criteria were as follows: (1) presence of other neurological diseases that can cause brain dysfunction; (2) presence of systemic diseases that can cause cognitive impairment; (3) presence of a condition that makes the patient unable to cooperate with the completion of cognitive tests; (4) presence of contraindications to MRI; (5) presence of mental retardation or neurological impairment; and (6) refusal to provide a blood sample.

Quality control in clinical research

Inclusion and exclusion criteria were established to minimize bias in the clinical studies. The healthy control group comprised patients screened at the Health Examination Center of the Zaozhuang Hospital of Traditional Chinese Medicine. Patients in the control group were required to have a CDR of 0, and were similar to patients in the AD group in terms of age, sex, HAMA, and HAMD scores. Brain information was collected using 1.5T/3.0T

MRI, and MR reports were issued by two professional doctors in the radiology department of Zaozhuang Hospital of Traditional Chinese Medicine. The participants and their families were assessed by a qualified neuropsychologist in a quiet setting, followed by screening by two neurologists (including at least one lead physician) according to the inclusion and exclusion criteria. After participants were enrolled, venous blood was collected by a nurse, and peripheral blood mononuclear cells (PBMCs) were extracted.

The study was conducted in accordance with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Zaozhuang Hospital of Traditional Chinese Medicine (2025-syyws-001).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 20 PBMC samples, from ten patients with AD and ten healthy controls using TRIzol reagent according to the manufacturer’s instructions. RNA (1 µL) was detected with a nanometer photometer N50. Reverse transcription of mRNA was performed using a SureScript First-Strand cDNA Synthesis Kit (GeneCopoeia Inc., Rockville, MD, USA). Amplification and dissolution curves were drawn, and cycle threshold (Ct) values were determined. The primer sequences are listed in Table 1. Finally, the relative expression was calculated using the 2^{-ΔΔCt} method. Finally, the 2^{-ΔΔCt} value was calculated and the p-value was calculated using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).The method refers to previous studies³⁰.

Statistical analysis

All statistical analyses were performed using R language (version 4.3.2). SPSS (version 19.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 9.4.1; GraphPad Software, San Diego, CA, USA) were used to perform statistical analyses and data mapping. The statistical significance of the differences in sex between groups was assessed using Fisher’s exact test. Age, CDR score, HAM-A score, HAM-D score, and relative mRNA expression were normally distributed, and an independent sample t-test was used. P-values (*) <0.05 were considered statistically significant, with p-values (**) <0.01 flagged as highly significant.

Results

Preliminary processing of single-cell expression profile data

The single-cell expression profiles used in this analysis included 25 AD samples and 24 control samples. The DoubletFinder package was used to filter doublet cells, and the feature expression levels of 178,303 cells were included for subsequent analysis (Supplementary Fig. 1A,B). The ten genes with the highest standard deviations are displayed (Supplementary Fig. 1C).

Single-cell data cell subpopulation annotation

The optimal number of principal components (PCs) was observed through ElbowPlot: 20 (Supplementary Fig. 1); principal component analysis (PCA) dimensionality reduction analysis revealed a batch effect between samples (Supplementary Fig. 1E). Harmony analysis was further used to reduce the dimensionality and remove the batch (Supplementary Fig. 1F), and 17 subtypes were obtained through UMAP (Fig. 1A). We further annotated each subtype, and 17 clusters were annotated to nine cell categories, including astrocytes, endothelial cells, smooth muscle cells, microglia, inhibitory neurons, excitatory neurons, oligodendrocytes, oligodendrocyte precursor cells, and unknowns (Fig. 1B), bubble diagrams of the classic markers of the 9 cell types (Fig. 1C), and grouping proportions (Fig. 1D).

Primer name	Sequence (5' to 3')
H- BTG1-187F	CAAGTTTCTCCGCACCAAGG
H- BTG1-187R	CTGTGCTGCCTGTCCAATCA
H-EPB41L4A-90F	GCAGGTGAAGCAAGATGTCC
H-EPB41L4A-90R	AGCTCCGACTGGATGGCATA
H-HERPUD1-71F	CAGCCCCATTTCACAACCAGT
H-HERPUD1-71R	AACCACTTGAGGAGCAGCATT
H-SLC3A2-92F	GGTTTTCTCACCCAGTGCAT
H-SLC3A2-92R	GGTTAGAGGGTAGGCAGTCG
H- SLC7A11-138F	TCTTTGTGTCCTCTCTGC
H- SLC7A11-138R	GTAGAGGAGTGTGCTTGCGG
H- SLC7A 5-250F	ATCGGGAAGGGTATGTGTC
H- SLC7A 5-250R	TGGACAGGGTGGTGAAGTAGG
H-β-actin-F	AGCCATGTACGTAGCCATCC
H-β-actin-R	CTCTCAGCTGTGGTGGTGAA

Table 1. Primers.

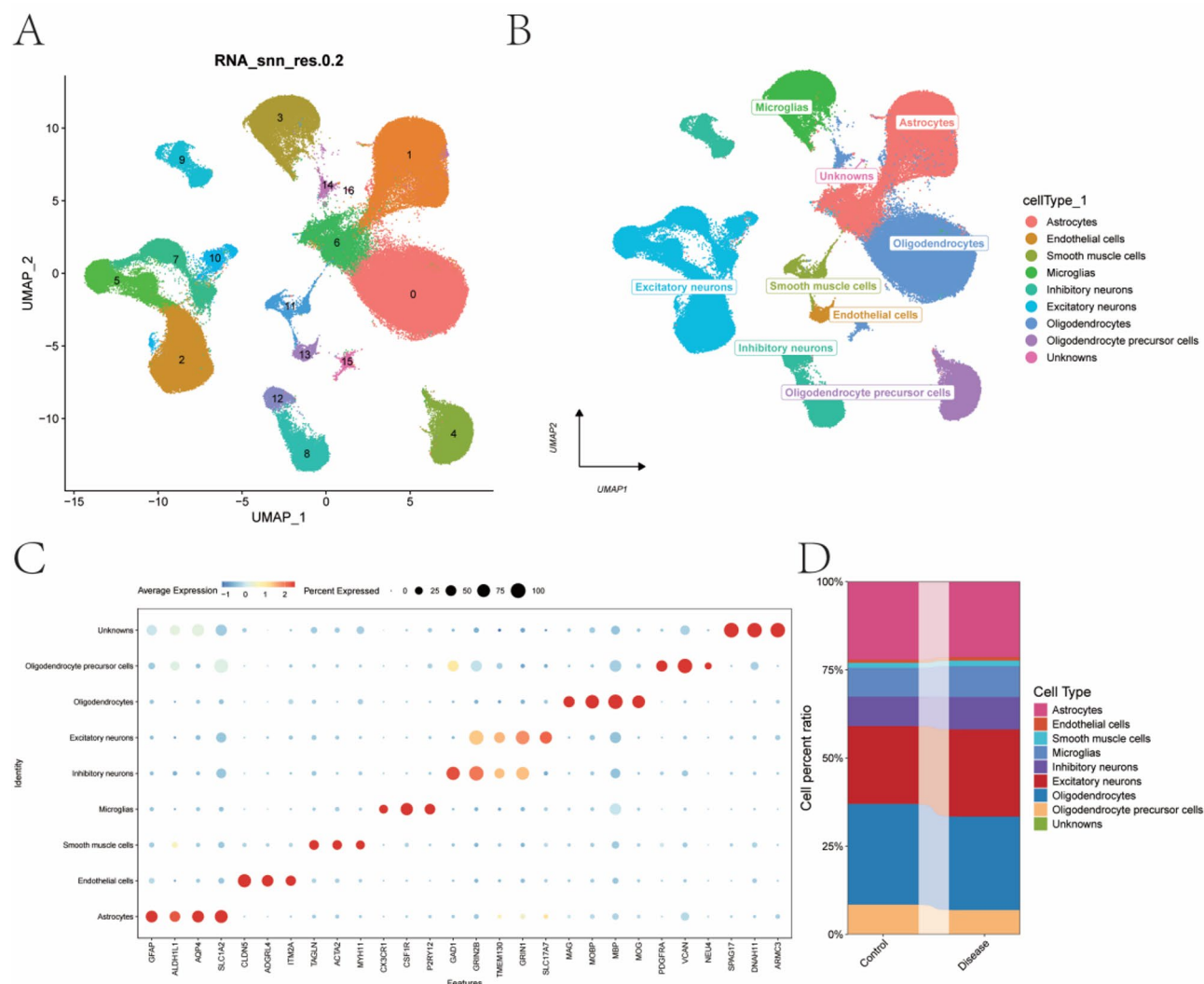


Fig. 1. Annotation of cells. **(A)** Based on the important components available in principal component analysis (PCA), we divided the cells into 17 clusters through the UMAP algorithm. **(B)** Cell annotation situations of 17 clusters. The 17 clusters were annotated into nine cell types, namely astrocytes, endothelial cells, smooth muscle cells, microglia, inhibitory neurons and excitatory neurons, oligodendrocytes, oligodendrocyte precursor cells, and unknowns. **(C)** Bubble plots of dotplots for nine types of cells and cell markers. **(D)** The differences in the proportion of nine types of cells in the two groups of samples.

Quantitative integrated stress response scores and differential analysis of high and low scores and cellchat

We then obtained 129 genes related to the integrated stress response from the literature³¹ and used AUCell to quantify the integrated stress response genes in single cells. The integrated stress response score of endothelial cells was significantly higher in the disease group than in the control group, which was used as the key cell subtype (Fig. 2A), and endothelial cells were extracted for differential analysis (Fig. 2B). The screening conditions were $p_{\text{val_adj}} < 0.05$, $|\log_2| > 0.25$, and 202 differentially expressed genes were identified (Table PbmMarkers-all.txt). We used the software package CellChat to analyze the ligand-receptor relationship of the features in the single-cell expression profiles of these two groups. We observed complex interactions between these cell subtypes (Fig. 2C). The ligands of High_Endothelial cells and Low_Endothelial cells are shown (Fig. 2D).

LASSO regression and random forest feature selection

To further identify the key genes that affect AD among the integrated stress-related genes, the 202 differentially expressed genes obtained in the previous step and the integrated stress-related genes were intersected to obtain 11 genes (Fig. 3A). Characteristic AD genes were screened using LASSO regression and random forest. LASSO regression identified seven genes as characteristic genes of AD (Fig. 3B,C); however, we screened the characteristic genes in AD through random forest, and took the top ten genes as the characteristic genes of AD (Fig. 3D). We then took the intersection with the characteristic genes screened by the LASSO regression

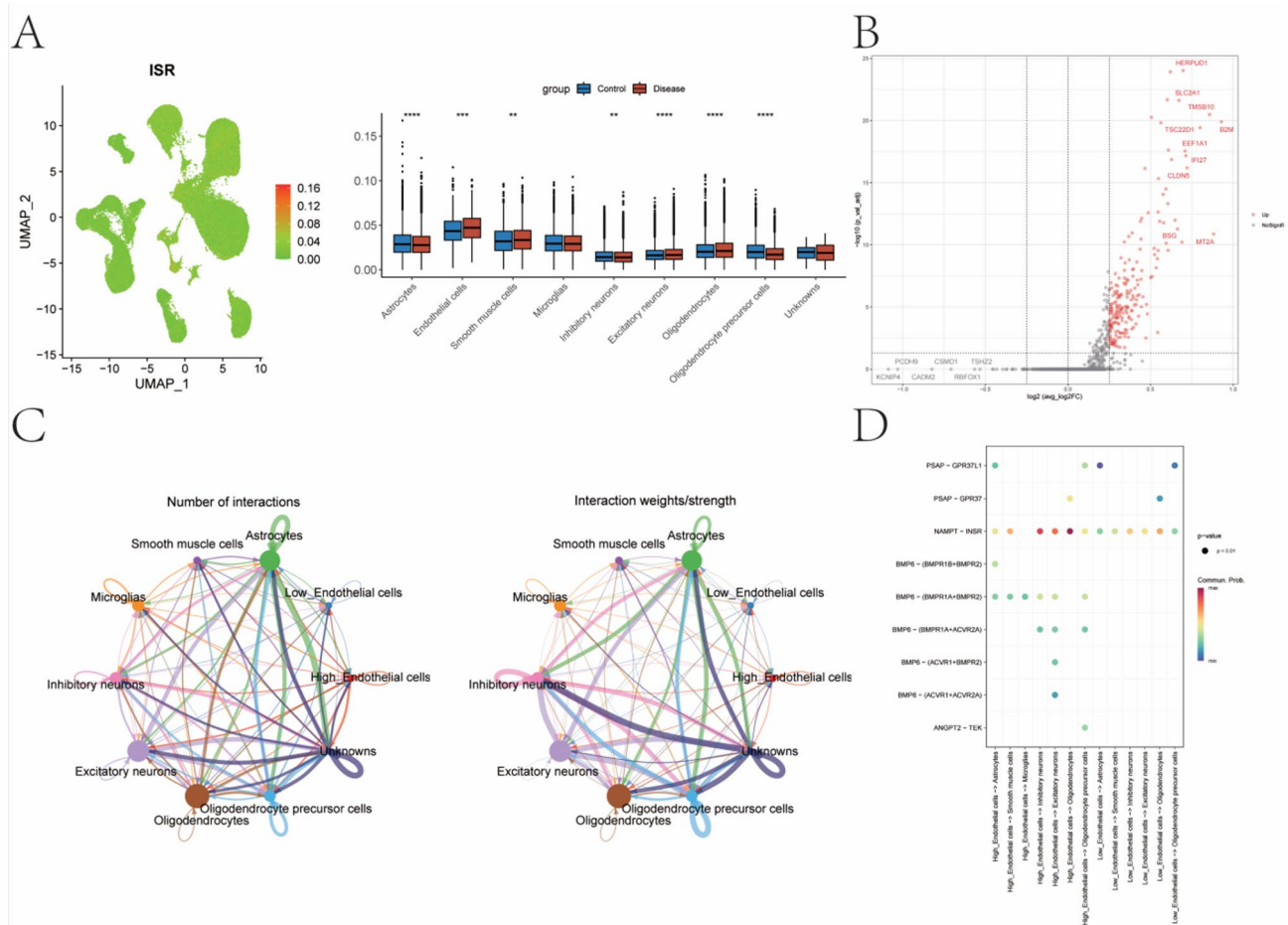


Fig. 2. Differences in ISR scores and cell communication. **(A)** The differences in ISR scores and the nine types of cells between the control and disease. Red represents the disease group score, and blue represents the control group score (ISR: Integrated stress score). **(B)** Volcano plots of differentially expressed genes, with blue indicating differentially downregulated genes and red indicating differentially up-regulated genes. **(C)** The cell interaction networks among nine types of cells, with the edge width representing the communication probability and intensity between cells. **(D)** Bubble diagrams of receptors and ligands between cells. In the figure, the communication intensity is represented by the color gradient (red to blue), and the bubble size reflects the significance. Among them, the ligand-receptor pairs marked by the red bubble (such as NAMPT-INBR) suggest the key intercellular regulatory relationship. The arrow direction indicates the signal transmission path, while the marked p-value and threshold line (such as $x + 0.5$) are used to determine the statistical significance of the interaction.

algorithm, and screened out six intersection genes in total (Fig. 3E), which were used as key genes for subsequent research: *BTG1*, *EPB41L4A*, *HERPUD1*, *SLC3A2*, *SLC7A11*, and *SLC7A5*.

Signaling pathways involved in key genes

Next, we studied the specific signaling pathways involved in key genes and explored the potential molecular mechanisms through which these genes affect disease progression. The GSEA results showed that *BTG1* was enriched in signaling pathways such as the NF-kappa B, TNF, and IL-17 signaling pathways (Fig. 4A). *EPB41L4A* was enriched in signaling pathways such as the PPAR, NF-kappa B, and Notch signaling pathways (Fig. 4B). *HERPUD1* was enriched in signaling pathways such as the NF-kappa B, IL-17, and p53 signaling pathways (Fig. 4C). *SLC3A2* was enriched in signaling pathways such as the NF-kappa B, TNF, and PPAR signaling pathways (Fig. 4D). *SLC7A11* was enriched in signaling pathways such as the PPAR, Notch, and NF-kappa B signaling pathways (Fig. 4E). *SLC7A5* was enriched in signaling pathways such as the PI3K-Akt, NF-kappa B, and TGF-beta signaling pathways (Fig. 4F). The GSEA results were shown in Fig. 5. *BTG1* was enriched in signaling pathways such as IL6_JAK_STAT3_signaling and P53_pathway. *EPB41L4A* was enriched in signaling pathways such as INTERFERON_ALPHA_RESPONSE and EPITHELIAL_MESENCHYMAL_TRANSITION. *HERPUD1* is enriched in signaling pathways such as ANDROGEN_RESPONSE and WNT_BETA_CATENIN_signaling. *SLC3A2* is enriched in signaling pathways such as ESTROGEN_RESPONSE_LATE and INTERFERON_GAMMA_RESPONSE. *SLC7A11* is enriched in signal pathways such as COAGULATION and NOTCH_signaling. *SLC7A5* is enriched in signal pathways such as APICAL_JUNCTION and HYPOXIA.

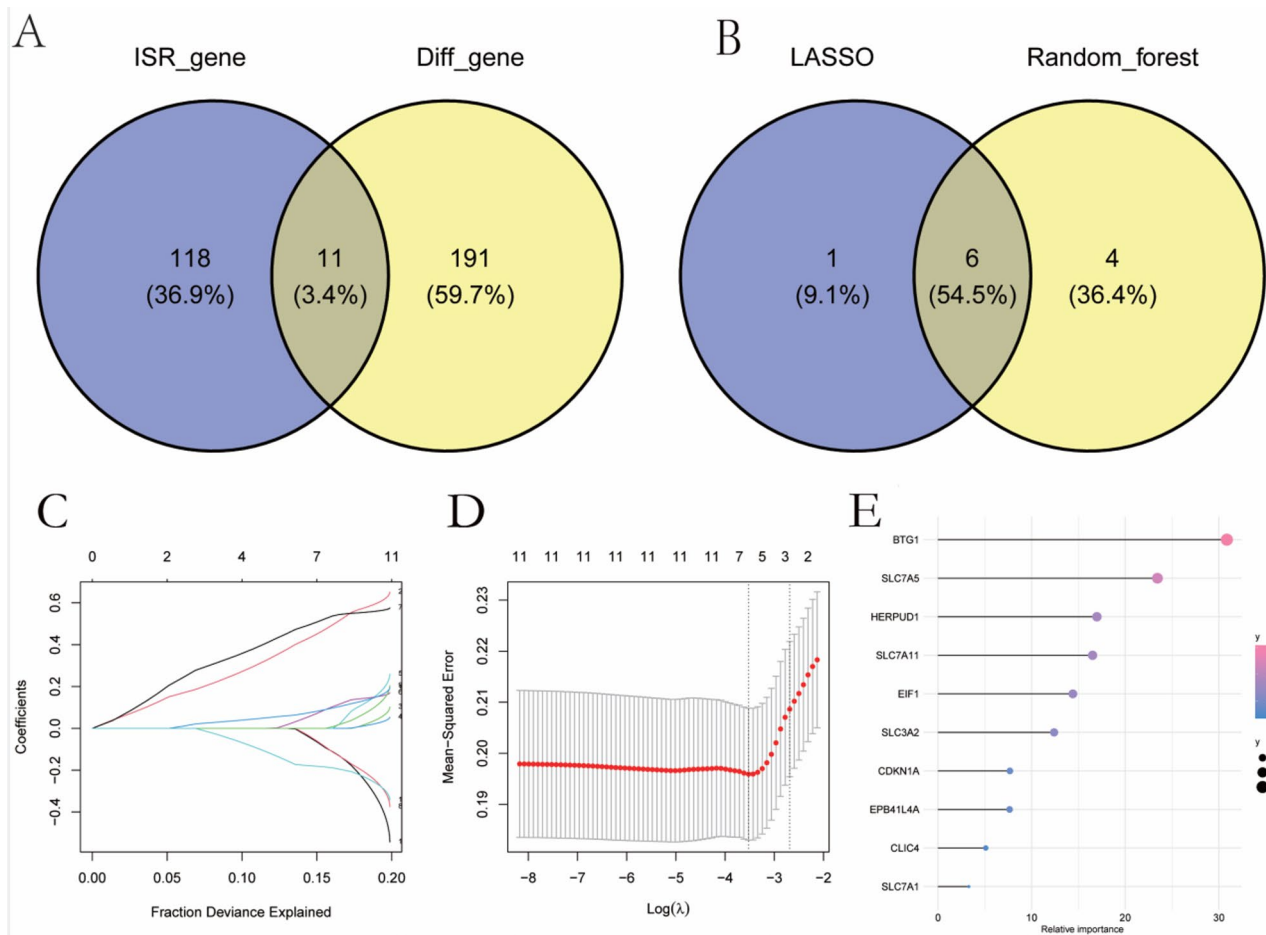


Fig. 3. Construction of LASSO and random forest models. **(A)** Venn diagram of the intersection of ISR genes and differentially expressed genes. **(B)** Take the intersection of the LASSO regression algorithm and the characteristic genes screened out by the random forest. **(C)** The distribution of LASSO coefficients and the gene combination at the minimum lambda value. **(D)** Ten cross-validations for the selection of tuning parameters in the LASSO model to determine the minimum lambda value. **(E)** Characteristic genes of the random forests.

Immune infiltration analysis

The microenvironment is mainly composed of fibroblasts, immune cells, the extracellular matrix, multiple growth factors, inflammatory factors, and unique physical and chemical characteristics. The microenvironment significantly affects the diagnosis, survival outcomes, and clinical treatment sensitivity of the disease. We demonstrated the distribution of immune cell infiltration levels and immune cell correlations in different forms (Fig. 6A, Supplementary Fig. 2). The levels of macrophages M2, neutrophils, and CD4 resting memory T cells in the disease group were significantly higher than those in the control group, while the levels of activated Natural killer (NK) cells and follicular helper T cells were significantly lower (Fig. 6B). We further explored the relationship between key genes and immune cells and found that *BTG1* was significantly positively correlated with monocytes and neutrophils, and significantly negatively correlated with memory B cells and activated NK cells (Fig. 6C). *EPB41L4A* was significantly positively correlated with macrophages M2 and eosinophils, and significantly negatively correlated with follicular helper T cells and macrophages M0 (Fig. 6D). *HERPUD1* was significantly positively correlated with CD4 resting memory T cells and neutrophils, and significantly negatively correlated with naive CD4 T cells and activated NK cells (Fig. 6E). *SLC3A2* was significantly positively correlated with CD4 resting memory T cells and macrophages M1, and significantly negatively correlated with CD8 T cells and follicular helper T cells (Fig. 6F). *SLC7A11* was significantly positively correlated with macrophages M2 and activated dendritic cells, and significantly negatively correlated with follicular helper T cells and macrophages M0 (Fig. 6G). *SLC7A5* was significantly positively correlated with CD4 resting memory T cells and neutrophils, and significantly negatively correlated with CD8 T cells and dendritic cells (Fig. 6H). We also conducted co-expression analyses of key genes and different immune factors, including immunosuppressive factors, immunostimulatory factors, chemokines, and receptors. These analyses suggested that the key genes are closely related to the level of immune cell infiltration (Fig. 7A–E).

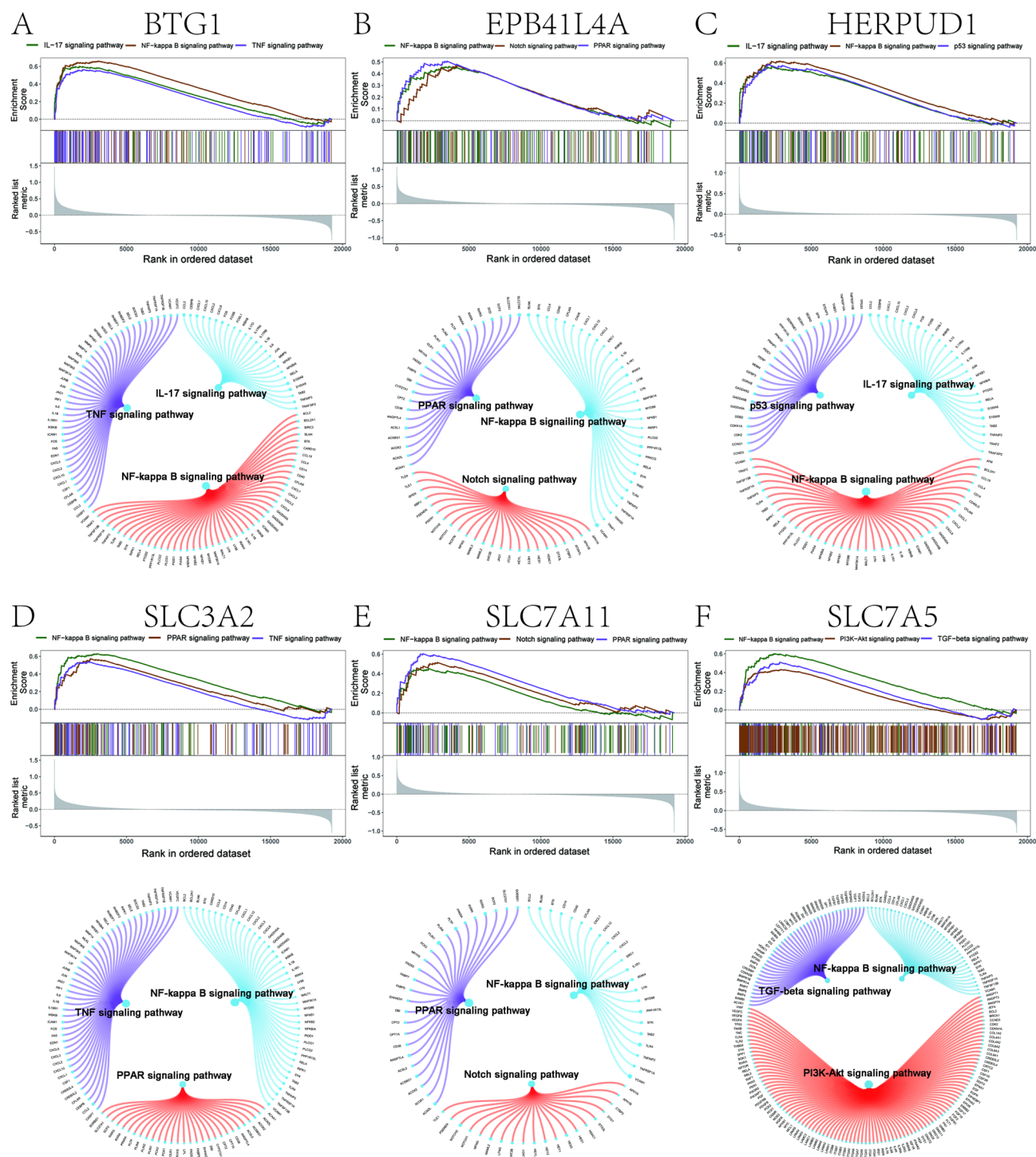


Fig. 4. GSEA analysis of key genes. (A–F) The KEGG signaling pathways involved in the key genes, as well as the pathway regulation and the genes involved. KEGG is developed by Kanehisa Laboratories. Reference: Kanehisa, M., Furumichi, M., Sato, Y., Matsuura, Y. and Ishiguro-Watanabe, M.; KEGG: biological systems database as a model of the real world. *Nucleic Acids Res.* 53, D672–D677 (2025). <https://doi.org/10.1093/nar/gkae909>.

Relationship between key genes and disease-related genes

Disease regulation-related genes were identified using the GeneCards database (<https://www.genecards.org/>). The expression levels of 20 genes ranked high in Relevance*score and expressed in the transcriptome were analyzed, and it was found that the expression of *A2M*, *APP*, *BACE1*, *GRN*, *MAPT*, *PLAU*, *PRNP*, *PSEN2*, *SORL1*, *TARDBP*, *TOMM40*, *TREM2*, *VCP* was significantly different between the two groups of patients. We then performed a correlation analysis of the key genes and disease-regulated genes. The expression levels of key

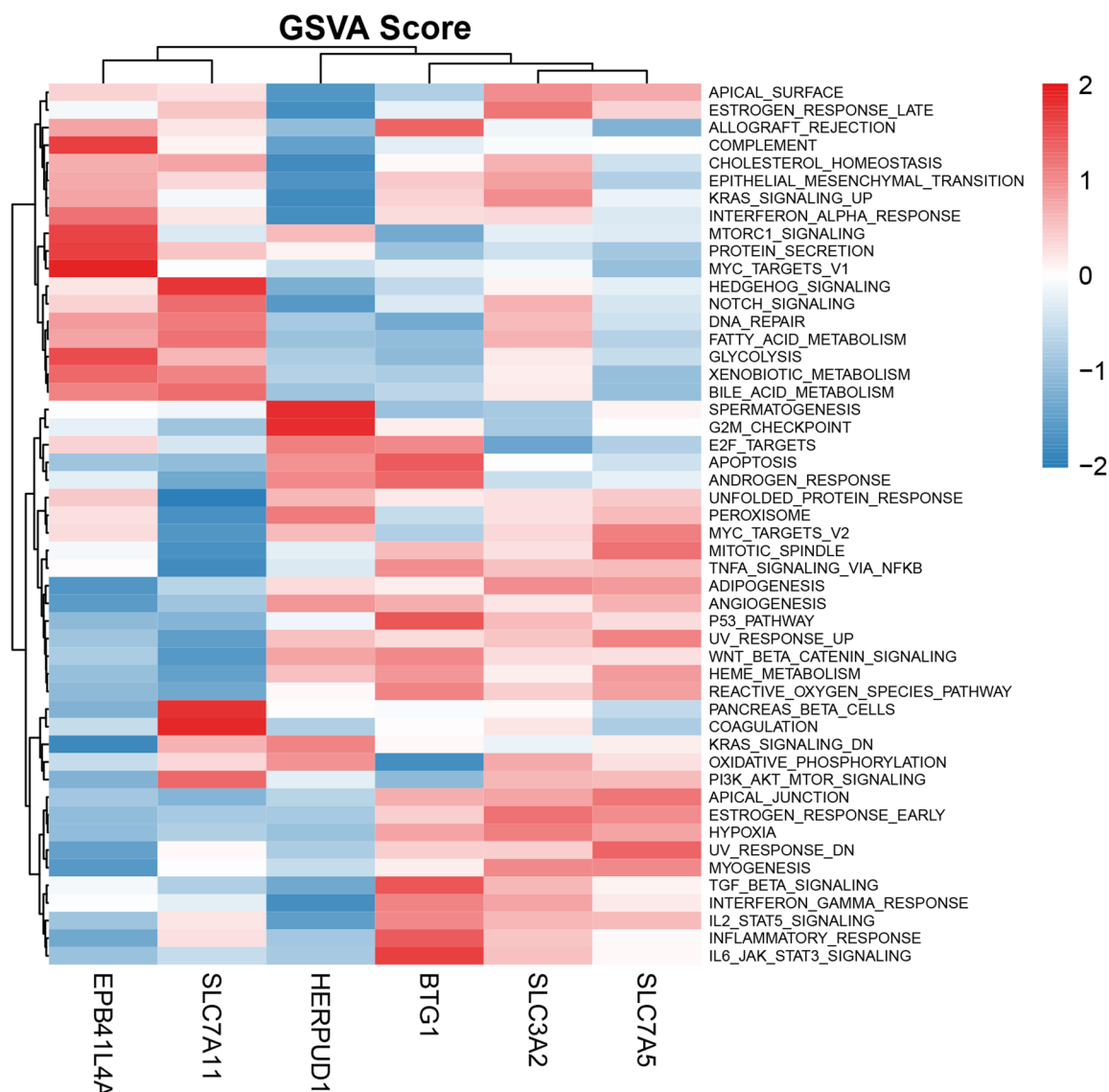


Fig. 5. Analysis of key gene GSVA. GSVA analysis of key genes: Blue indicates the signaling pathways involved in low expression of genes, red indicates the signaling pathways involved in high expression genes.

genes were significantly correlated with those of the disease-regulated genes. Among them, *SLC7A11* and *APOE* were significantly positively correlated ($\text{cor} = 0.732$), whereas *SLC3A2* and *PRNP* were significantly negatively correlated ($\text{cor} = -0.494$) (Fig. 8).

Key gene-related clinical importance scores and nomogram model

We analyzed the relevant clinical importance scores of key genes through the official AlzData website, obtained supplementary CFG scores and rankings, and displayed single-cell RNA sequencing of key genes in different cell types of the human brain (Fig. 9A–F). We present the results of the regression analysis in the form of a nomogram based on the expression of key genes. The results of the regression analysis showed that, in all of our samples, the values of different clinical indicators of AD and the distribution of key gene expression showed varying degrees of contribution in the entire scoring process (Fig. 10A). The nomogram model had good predictive performance, and the ROC curve was drawn (Fig. 10B).

Expression profile of key genes in single cell data and immunometabolic pathways

In this study, we analyzed the expression of key genes in single cells and demonstrated the expression of *BTG1*, *EPB41L4A*, *HERPUD1*, *SLC3A2*, *SLC7A11*, and *SLC7A5* in astrocytes, endothelial cells, smooth muscle cells, microglia, inhibitory neurons, excitatory neurons, oligodendrocytes, oligodendrocyte precursors, cells, and unknowns (Fig. 11A, B). We used AUCell to quantitatively score immune metabolism-related pathway genes in single cells and employed bubble charts to display the expression differences of key genes in immune metabolism-related pathways³². The results showed that *HERPUD1* was enriched in pathways such as hedgehog

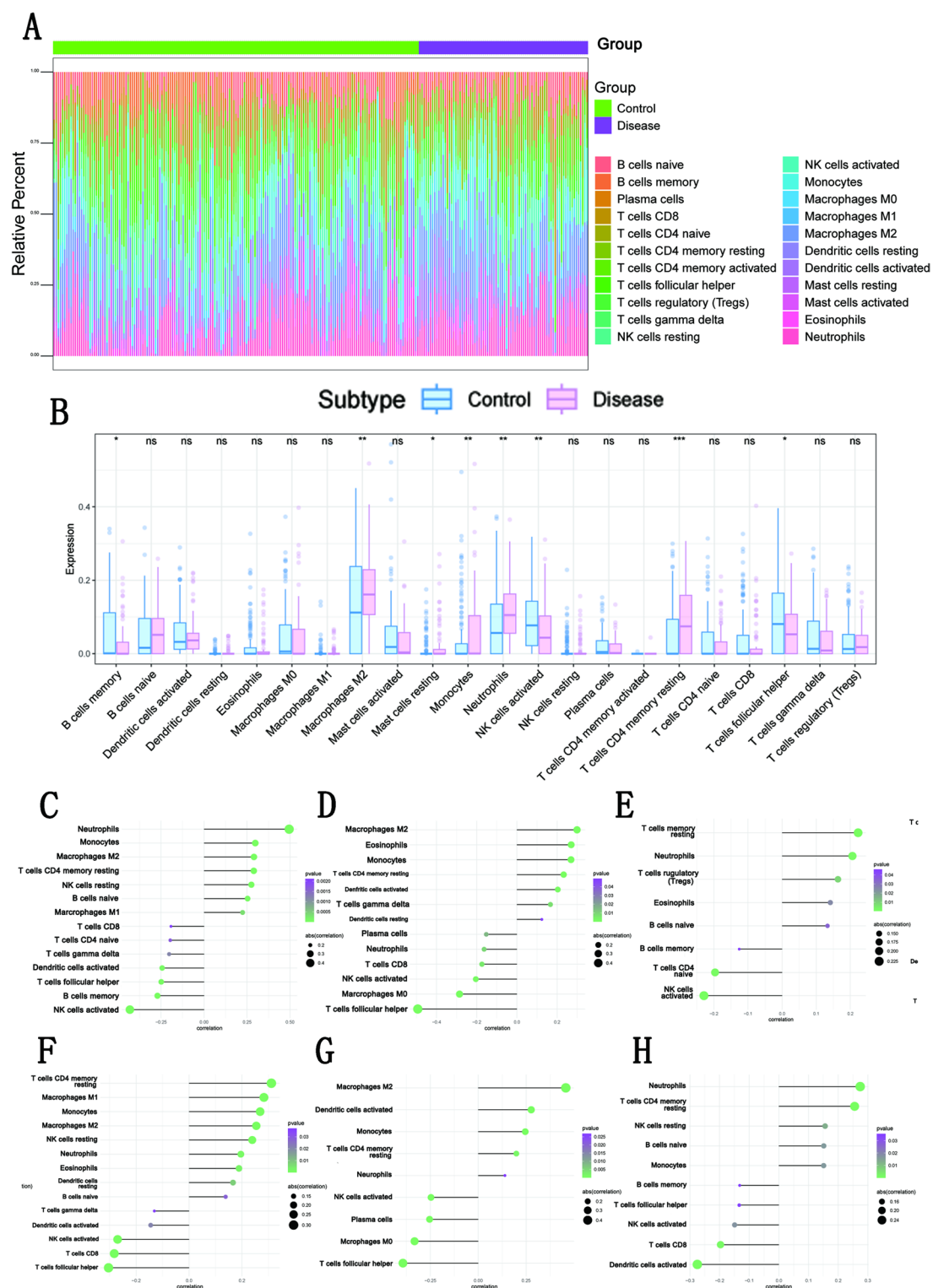


Fig. 6. Analysis of immune infiltration. (A) The vertical axis represents the relative percentage of immune cell subsets, and the horizontal axis represents different samples. (B) The difference in immune cell content between the control and disease samples. (C–H) The correlation between the key genes and immune cells. The larger the point is, the greater the correlation is. The greener the point is, the smaller and more significant the p-value is.

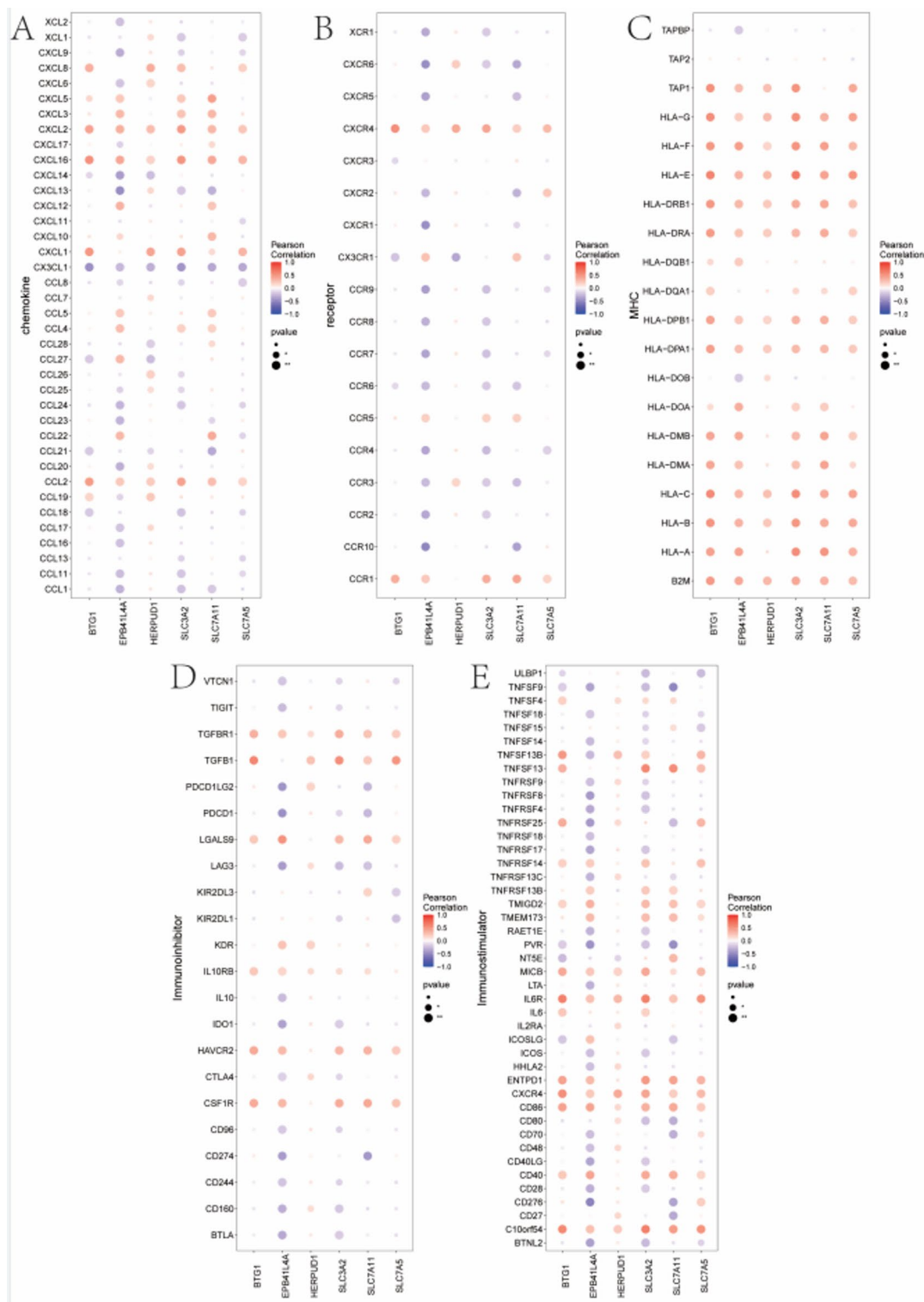


Fig. 7. Correlation between key genes and immune factors. (A–E) The correlations of key genes and chemokines, immunoinhibitors, immunostimulators, MHC, and receptors. Red represents a positive correlation, blue represents a negative correlation, and the larger the dot, the greater the p-value.

and hedgehog signaling; *SLC7A5* was enriched in hedgehog signaling; *EPB41L4A* was enriched in pathways such as hedgehog_signaling; *BTG1* was enriched in pathways such as hedgehog_signaling; *SLC7A11* was enriched in pathways such as notch_signaling; and *SLC3A2* was enriched in pathways such as hedgehog_signaling (Fig. 11C).

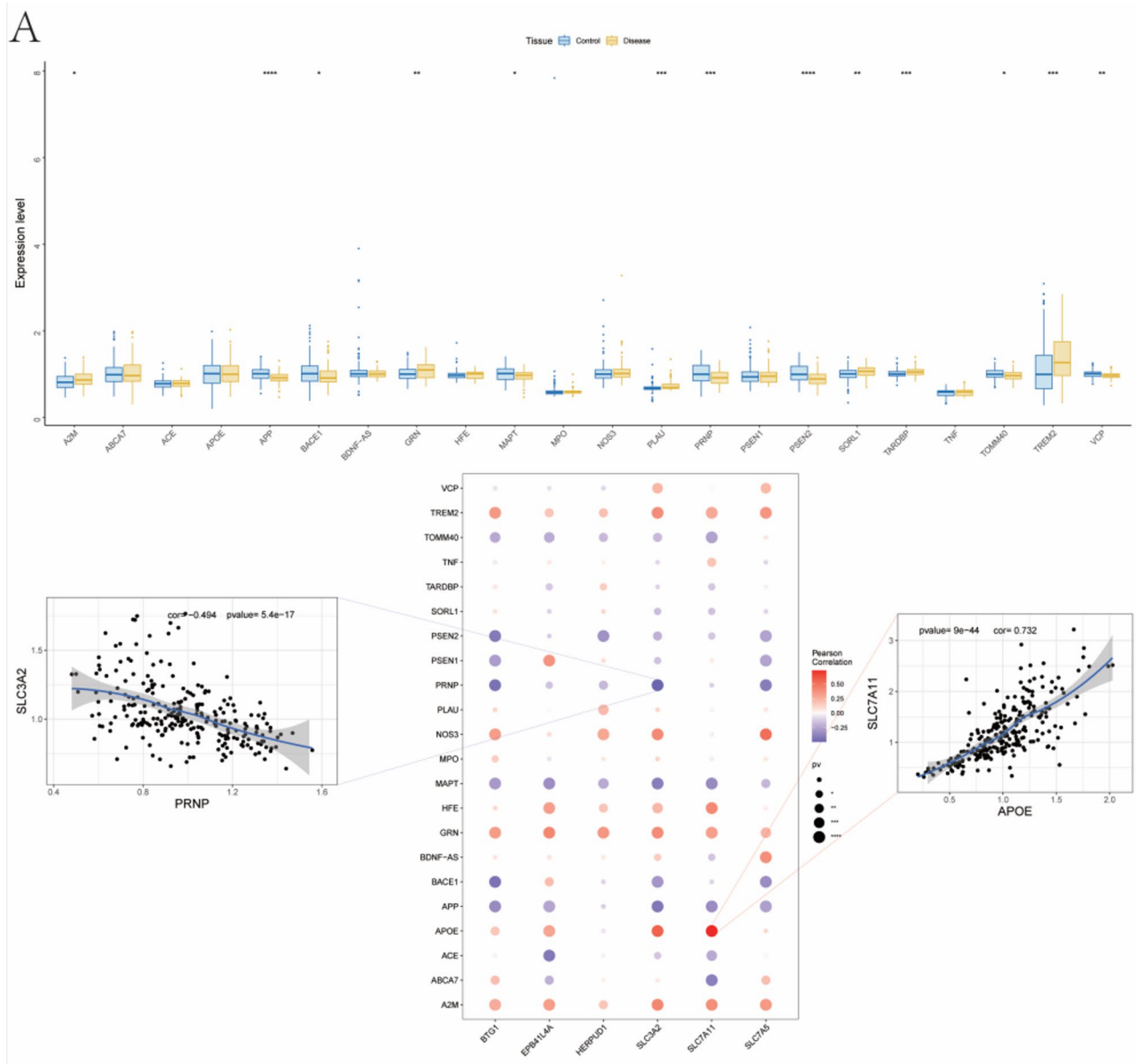


Fig. 8. Disease correlation. (A) Expression differences of disease regulatory genes. Blue represents control patients, and yellow represents disease patients. (B) Correlation analysis between key genes and disease genes. Blue indicates a negative correlation, and red indicates a positive correlation.

Pseudo-chronological analysis of key cells

Endothelial cells were extracted in the next step of analysis. First, we calculated the similarity between the cells and constructed a cell differentiation trajectory. Subsequently, by visualizing the trajectory, a picture of the cell differentiation trajectory constructed over time can be generated to show the developmental process of the cells, which can be used to study the process of cell differentiation and gene expression patterns at different time points. The pseudotime values are output. Pseudotime is the probability calculated by monocle2 based on cell gene expression information, indicating the order of time. Images of cells colored according to cell type (Fig. 12A,B). These genes were differentially expressed in different branches. However, this cannot be displayed on the overall heat map. We selected all branch points, identified genes with large differences in cell expression before and after the branch point, and visualized them in a branch heat map. The trajectory starts from the middle and differentiates to both sides, representing the differences at both ends of the branch points respectively. The pre-branch of the Cell Type in the legend represents the data before the branch point. Cell fate1 is the side with the smallest state after the branch point. Cell fate2 is the side with the largest state after the branch point. The default classification was based on gene changes. Six clusters (Fig. 12C) showed gene expression changes in key genes from the beginning to the end of the pseudo-time course (Fig. 12D).

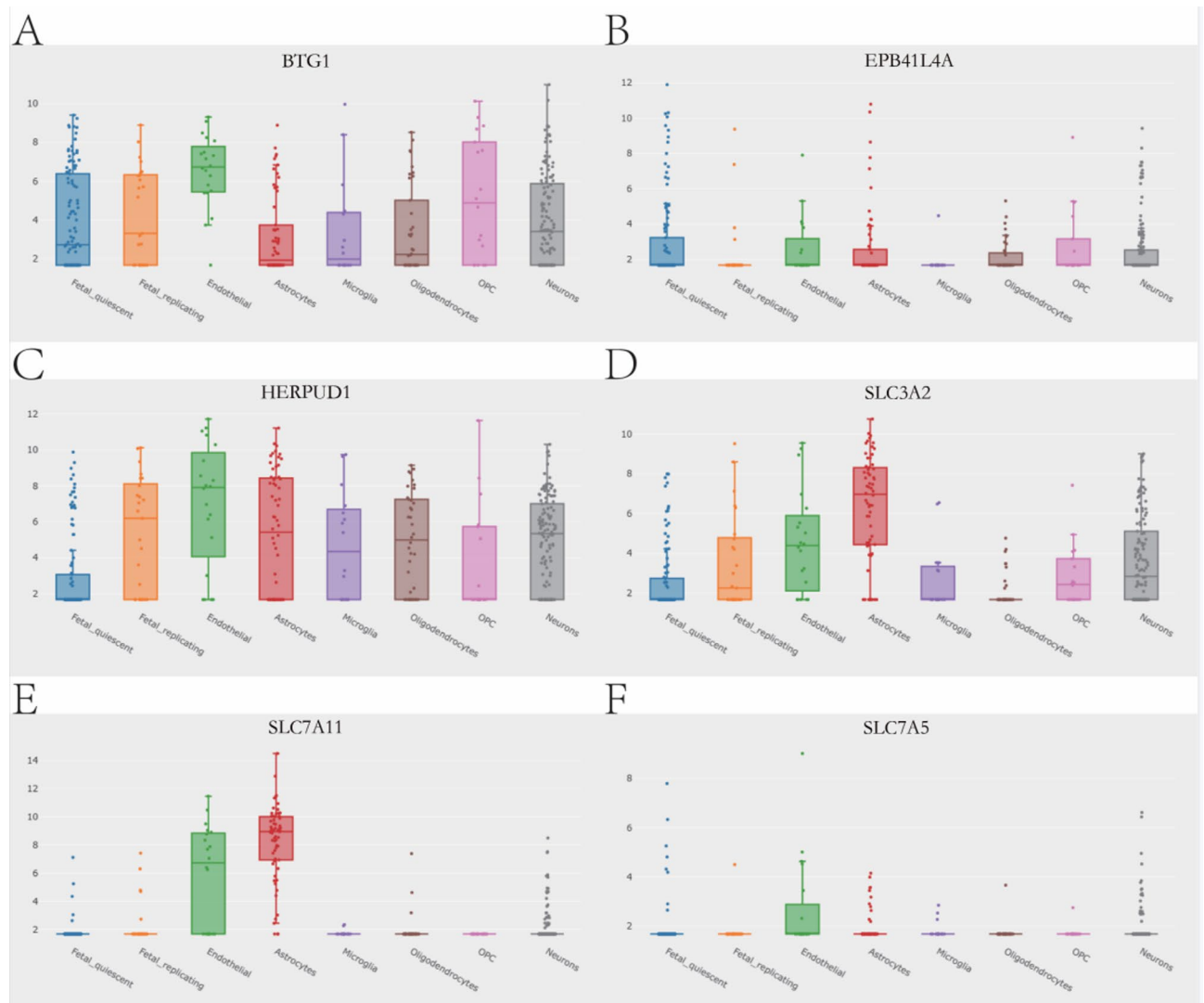


Fig. 9. Clinical importance score related to key genes. (A–F) Clinical importance scores related to key genes predicted based on the AlzData database. The vertical coordinate represents the expression of genes.

Validation of key gene expression in patients with AD

To further verify the changes in the expression levels of key genes in the AD population, we screened and tested patients who visited the hospital and healthy controls who underwent physical examinations at the physical examination center. Ten patients with AD and ten healthy controls were screened. The baseline information is shown in Table 2. There were no significant differences between the two groups in terms of age, gender, HAMA score and HAMD score. The CDR score of AD patients was higher than that of the control group ($p^{**} < 0.01$). The RT-qPCR results (Fig. 13) showed that the relative expression level of *HERPUD1* mRNA in AD patients was higher than that in the control group ($p^{**} < 0.01$), and the relative expression level of *BTG1*, *EPB41L4A*, *SLC3A2*, *SLC7A11*, and *SLC7A5* mRNA in AD patients was lower than that in the control group ($p < 0.05$), which was consistent with the results of bioinformatics analysis.

Discussion

Neurodegenerative diseases involve a series of complex changes that occur at the molecular level during the evolution, development, maturation, and aging of neural tissue. Researchers do not fully understand this series of dynamic changes and their mechanisms, and effective treatment methods remain limited³³. AD progresses from undetectable brain changes in patients to severe memory and thinking disorders, ultimately leading to a decline in living ability, known as the Alzheimer's continuum³⁴. An increasing number of studies have shown that the occurrence, development, and phenotypes of AD are associated with genetics³⁵. Indeed, genetic susceptibility plays an important role in the occurrence and development of AD under the same environmental conditions³⁶. There are also studies that have found A β -protein-precursor (A β PP)-derived intraneuronal A β (iA β) accumulated to neuronal integrated stress response (ISR)-eliciting levels trigger AD, and iA β produced independently of A β PP drives the disease³⁷. Therefore, extensive research needs to be conducted to discover and

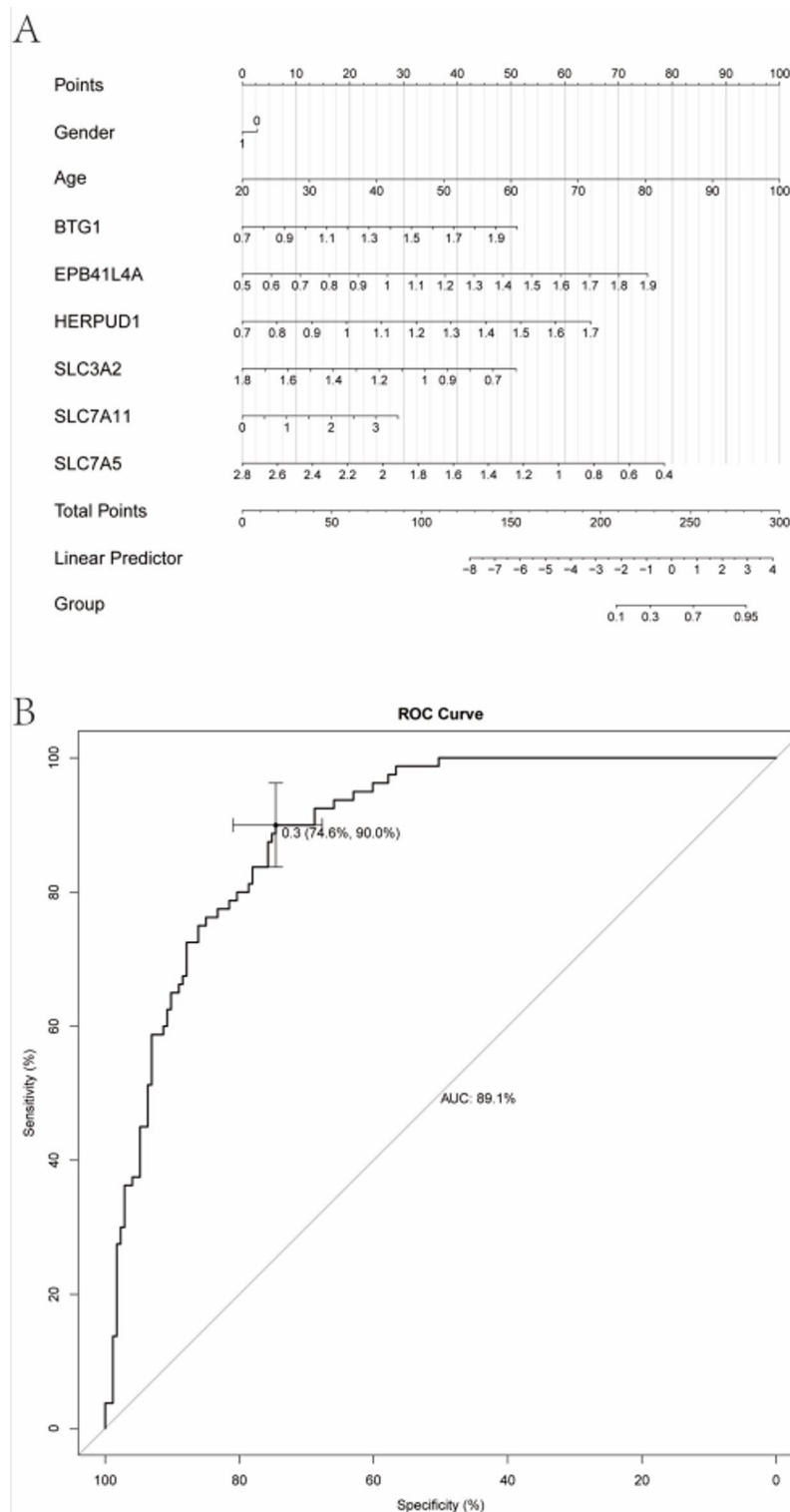


Fig. 10. Construction of nomogram models related to key genes. **(A)** Model-related nomogram of key genes and clinical characteristics. **(B)** ROC curve of the model.

explore key hub genes related to AD. With advancements in science and technology, bioinformatics, and the human genome, many AD-related genes have been discovered. However, due to differences in research methods, populations, disease classifications, and phenotypes, more rigorous studies are needed to verify the relevant conclusions. Currently, life science and computer analytical methods are widely applied in genetic analysis and screening.

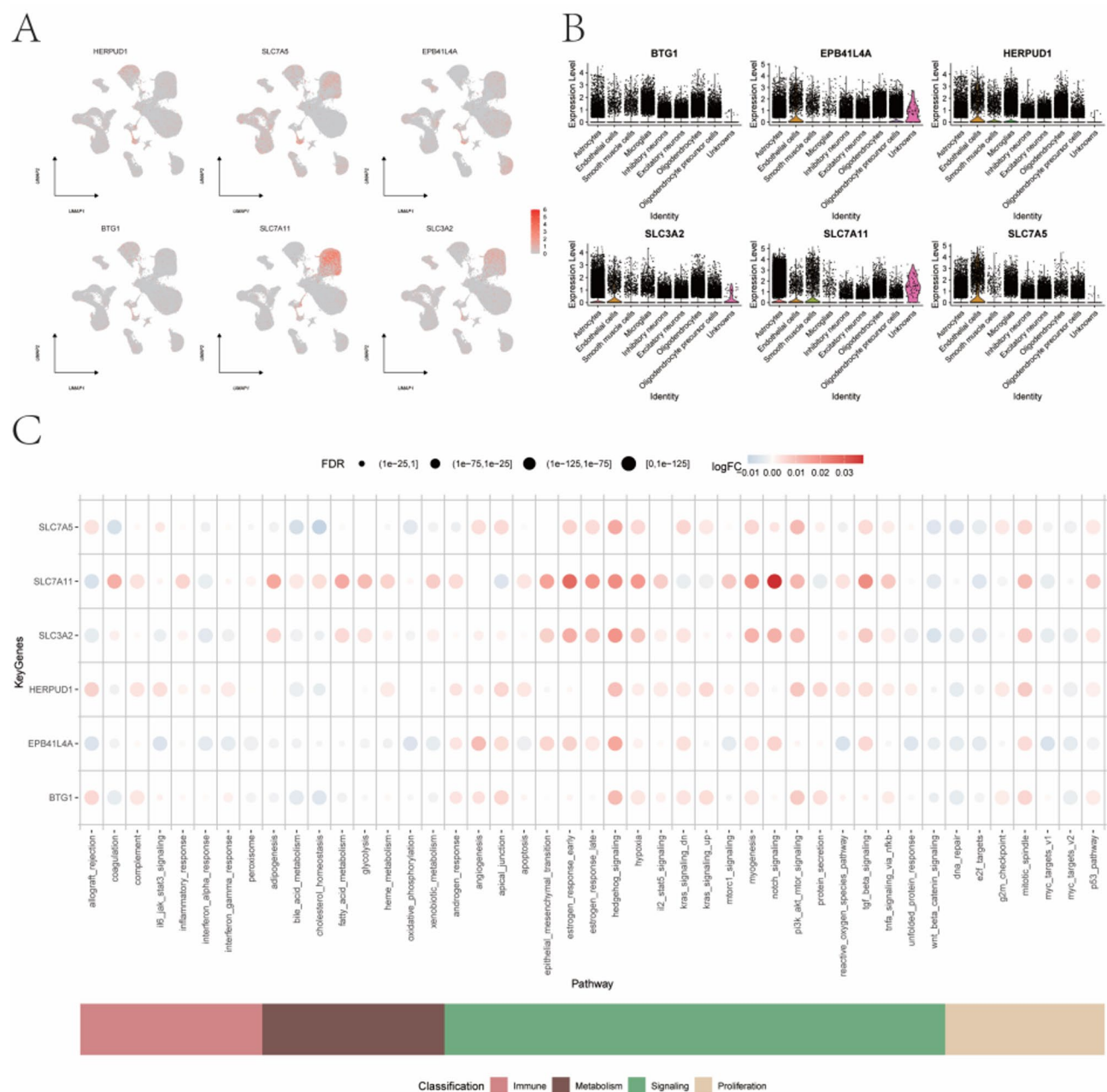


Fig. 11. Differences in the expression of key genes in single cells and the activity of immune metabolic pathways. (A) Scatter plot of the expression profile of key genes in a single cell. Orange-red indicates that the gene is expressed in this cell. (B) A violin plot of the expression profile of key genes in a single cell. One dot represents one cell. (C) Differences in the activity of key genes and immune metabolic pathways, with blue indicating low expression and red indicating high expression. The larger the dot, the smaller the p-value.

This study was based on the GEO database. Single-cell data (GSE264648) were downloaded. The integrated stress response genes were quantitatively scored in single cells using AUCell, and endothelial cells were determined as the key cell subtype. By taking the intersection of 202 differentially expressed genes and integrated stress-related genes, six AD-characteristic genes were screened through LASSO regression and random forest: *BTG1*, *EPB41L4A*, *HERPUD1*, *SLC3A2*, *SLC7A11*, and *SLC7A5*. Further verification was performed by collecting blood samples from clinical patients. The PCR results showed a gene expression trend consistent with the results of the bioinformatics analysis.

BTG1 is a negative regulator of the cell cycle that inhibits cyclin D1 and controls the transition from G1 to S³⁸. *BTG1* is essential for maintaining the dentate gyrus in the hippocampus. Knockout (KO) of the *BTG1* gene can present characteristics of early neurogenic aging. Adult *BTG1* KO mice showed impaired associative memory in the hippocampus. The increase in progenitor cell proliferation leads to the generation of new neurons, which integrate into existing memory circuits and enhance the performance of the hippocampus in

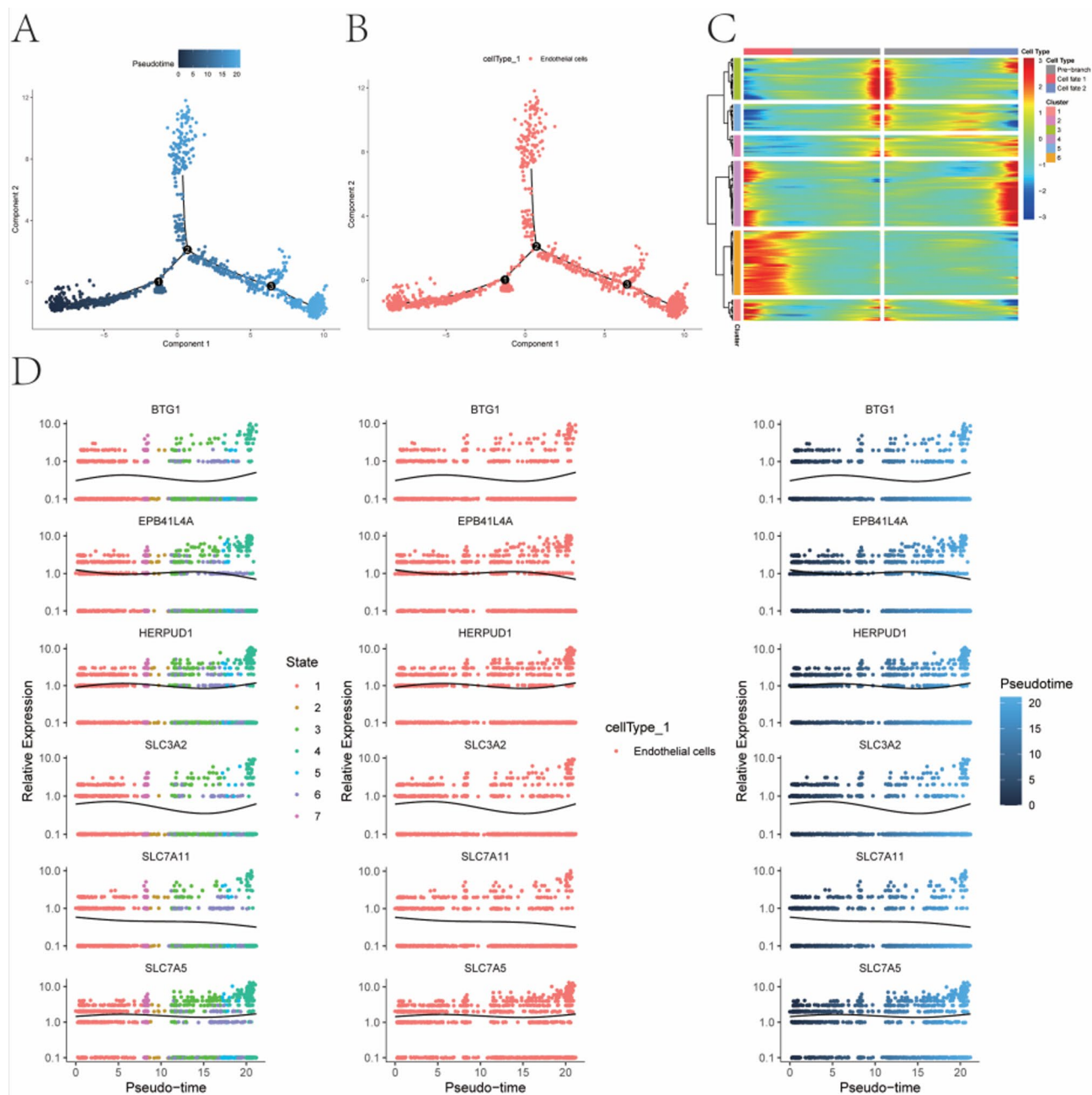


Fig. 12. Cell development trajectory. (A, B) Synchrony analysis and developmental trajectory of cells. (C) Gene expression dynamics of each pigment cell branch. (D) Relationship between the expression levels of key genes and the trajectory of cell development.

	AD group (n = 10)	Control group (n = 10)	P-value
Male	4	5	0.5
Female	6	5	
Age (mean $\pm$ standard deviation)	68.1 $\pm$ 2.65	65.3 $\pm$ 2.39	0.39
HAMA (mean $\pm$ standard deviation)	2.7 $\pm$ 0.37	2.6 $\pm$ 0.31	0.84
HAMD (mean $\pm$ standard deviation)	3.1 $\pm$ 0.32	3.0 $\pm$ 0.39	0.85
CDR (mean $\pm$ standard deviation)	1.00 $\pm$ 0.13	0.00	0.00

Table 2. Baseline information analysis.

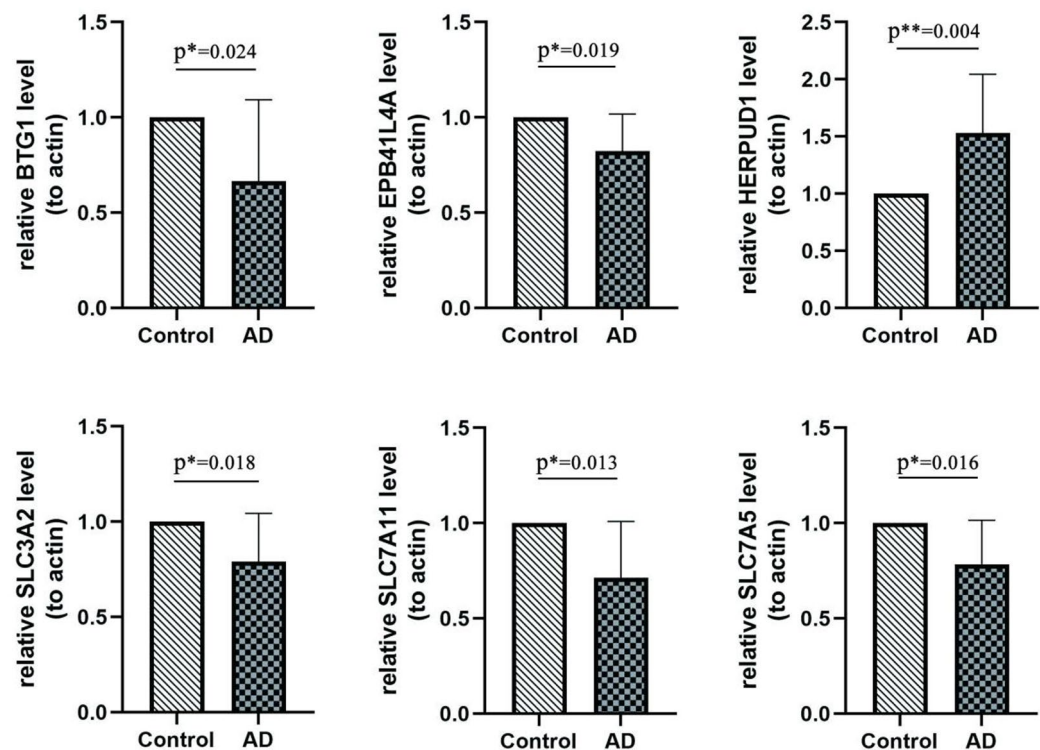


Fig. 13. Clinical sample validation. The quantitative reverse transcription polymerase chain reaction (RT-qPCR) results showed the comparison of the relative expression levels of mRNA of six key genes between AD patients and the healthy control group.

memory-dependent and learning tasks, including spatial memory and spatial pattern separation, novel object recognition, and situational fear-conditioned reflexes^{39–41}.

EPB41L4A is a long non-coding RNA (lncRNA). LncRNAs play an important role in the physiological and pathological processes of AD by interacting with transcription factors, epigenetic alterations, and the separation of RNA or proteins³⁹. Defective autophagy is a pathogenic factor of neurodegenerative diseases. In patients with AD, autophagy plays a role in clearing intracellular A β and MAPT/TAU tangles. Downregulation of *EPB41L4A* in patients with AD is associated with lower expression of multiple autophagy-related genes^{42–44}. This is achieved by reducing the expression level of histone acetyltransferase GCN5L2, thereby inhibiting the enrichment of acetylation, barbuylation, and lactation of these autophagy-related genomic proteins, and thus achieving the inhibition of autophagy-mediated A β clearance⁴⁵. The clearance and accumulation of A β -mediated by autophagy may be due to the dysfunction of the lysosomal pathway. When *EPB41L4A* is downregulated, the expression of the lysosomal markers *LAMP1*, *CTSB*, and *CTSD* is inhibited, suggesting that *EPB41L4A-AS1* plays a role in maintaining lysosomal function⁴⁶.

Homocysteine induces the conversion of endoplasmic reticulum protein (*HERPUD1*) into an ER-associated degradation complex (ERAD) with ubiquitin-like domain 1, which participates in the ubiquitination and relocalization of ERAD substrates^{47–50}. *HERPUD1* is involved in the regulation of inflammation-related diseases via ERS, including neurodegenerative changes caused by unknown neuroinflammation-related proteins⁵¹. In terms of regulating endothelial cell apoptosis, upregulation of *HERPUD1* promotes the induction of this injury event by angiotensin II⁵². In neurons and glioma cells, *HERPUD1* promotes cell survival under endoplasmic reticulum stress by inhibiting apoptosis^{53,54}. KO of *HERPUD1* can increase the expression of caspase-3, the most important terminal-cleaving enzyme during apoptosis, reduce the activity of caspase-12, and alleviate caspase-12-mediated apoptosis⁵⁵.

SLC3A2 is a type 2 transmembrane glycoprotein that forms disulfide-linked heterodimers with amino acid exchangers, including *SLC7A5* and *SLC7A11*⁵⁶, bringing N-glycans into the heterodimers. *SLC3A2* is involved in mediating cystine uptake in exchange for glutamic acid output, providing cysteine for the biosynthesis of glutathione (GSH), and alleviating oxidation-induced ferroptosis- or pyroptosis-induced cell death^{57–59}. *SLC7A5* balances essential intracellular amino acids and metabolic requirements. A recent study has shown that the absence of *SLC3A2* can lead to a serious imbalance in amino acid levels within cells; more specifically, it can cause a decrease in leucine concentration. Changes in *SLC3A2* levels may affect cell growth and survival by regulating leucine-mediated mTOR activation. The degradation of *SLC3A2* further activates the iron deposition pathway, promoting endothelial cells (EC) damage, and ultimately accelerating atherosclerosis. The regulation of system X_C⁻, involving *SLC3A2* and *SLC7A11*, plays an important role in regulating the intracellular transport of cysteine. Cysteine, as a substrate, supports GSH synthesis in cells. Activation of *SLC3A2* and *SLC7A11* enables

cells to restore REDOX homeostasis and maintain survival under stress conditions⁶⁰. Conversely, disrupting the XC-system can lead to glutathione depletion, endoplasmic reticulum stress, and ultimately, cell death⁶¹.

Through GSEA, it was found that the NF- κ B signaling pathway is a common molecular mechanism of these six characteristic genes. The NF- κ B signaling pathway is related to multiple mechanisms for the formation of AD, including the beta-amyloid protein cascade reaction, the neuroinflammatory hypothesis, and the gut microbiota theory. Studies have shown that peroxisome proliferator-activated receptor (PPAR γ) is a ligand-activated transcription factor, and NF- κ B is the key factor exerting the anti-inflammatory activity of PPAR γ ⁶². Experiments on A β overactivated microglia models and APPswe/PS1E9 transgenic mice revealed that PPAR γ reduced the number of activated microglia in mice by inhibiting the AMPK/NF- κ B pathway, suggesting that the NF- κ B signaling pathway is related to the deposition of A β ⁶³. NF- κ B proteins usually form dimer complexes, including an inhibitor of NF- κ B (I κ B) α and other related anchor protein repeat sequences. When cells are stimulated accordingly, I κ B kinase is phosphorylated, leading to the degradation of I κ B and the release of NF- κ B dimers, which then translocate into the cell nucleus. After the activated NF- κ B dimers enter the cell nucleus, they induce the transcription of related inflammatory mediators and immune genes, thereby promoting the expression of inflammatory factors and chemokines in cells, ultimately activating the inflammatory response^{64,65}. Inhibiting the NF- κ B-related signaling pathway can reduce the production of inflammatory factors and improve cognitive impairment in patients with AD⁶⁶. Some bacteria in the intestinal flora secrete large amounts of lipopolysaccharide (LPS). LPS is a potent inducer of the pro-inflammatory transcription factor NF- κ B(p50/p65) complex, which can bind to the TLR4 receptor and activate the downstream NF- κ B pathway, thereby releasing inflammatory cytokines, such as IL-1 and IL-6, ultimately leading to the occurrence and development of AD⁶⁷.

This study focused on immune infiltration in AD and presented the distribution of immune infiltration levels and the correlation between immune cells in different forms. These analyses suggest that the key genes are closely related to the infiltration level of immune cells and play an important role in the immune microenvironment. An increasing number of studies have found that AD is closely related to immune inflammation, providing a new direction and potential therapeutic target for the treatment of AD⁶⁸. The inflammatory response is a defense response of the body to abnormal substances, with the aim of eliminating harmful substances and promoting tissue repair. Multiple studies have suggested an important role of T cell immune infiltration in the occurrence and development of AD; that is, T cells enter the central nervous system through processes such as the blood-brain barrier from the peripheral circulatory system, triggering abnormal neuroimmune responses⁶⁹. Research on neurological diseases has shown that microglial activation in patients with AD damages blood vessels and parenchymal tissue. The use of immunosuppressive drugs may suppress brain inflammation, A β deposition, and tau hyperphosphorylation in AD. Further clarification of the immune mechanisms of AD may contribute to its treatment⁷⁰. Currently, many studies have been conducted on the mechanism, regulation, and potential therapeutic targets of immunity against AD. However, further studies are needed to clarify the detailed mechanisms and provide more accurate treatment ideas for clinical practice. Through immune infiltration analysis, we found that the infiltration degrees of macrophages M2, neutrophils, and CD4 resting memory T cells in AD were significantly higher than those in the control group, with key genes playing a role. *BTG1*, *EPB41L4A*, *HERPUD1*, *SLC3A2*, *SLC7A11*, and *SLC7A5* may be involved in the occurrence and development of AD through immune inflammation. However, there are currently no studies on key genes related to AD in the immune aspect. Therefore, it is worth exploring the related mechanisms of key genes in AD.

We analyzed the expression of key genes in single cells and quantitatively scored the genes related to immune metabolic pathways in single cells using AUCell. The results showed that the key genes were generally highly active in hedgehog-signaling and notch-signaling. Typical Hh signals are spontaneously initiated by active Hh ligands. After the mature Hh ligand binds to Ptch1/2⁷¹, it inhibits key protein kinases promoted by growth inhibitory specificity 1 (GAS1), oncogene (CDO), CDO sibling genes (BOC), and low-density lipoprotein receptor-related protein 2 (LRP2), including protein kinase A (PKA), casein kinase 1 (CK1), and synthase kinase 3 β (GSK3 β)^{72,73}, and deprives them of the ability to mediate the phosphorylation of the SUFU-Gli complex. Hh signals include PTCH-HH-mediated (Type I), SMO-dependent (Type II), and GLI-activated (Type III) signals⁷⁴. Ptch1 can regulate apoptosis by recruiting pro-apoptotic elements, such as caspase-9, dependent-related receptor transmembrane (DRAL), and tumor-upregulated caspase-9 antagonists (TUCAN) into its C-terminal domain. Most atypical Hh signaling axes do not rely on Ptch binding. By coupling SMO, a G protein-coupled receptor, to heterotrimeric G proteins of the G inhibition (Gi) family⁷⁵, the activities of several key protein kinases, including PKA, Ras homologous A (RhoA), RAS-related C3 botulinum toxin substrate 1 (Rac1), Neurofibromatosis type 1 (NF-1), and Src are altered. PI3K/PLC γ and calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2)^{76–79} promote changes in actin rearrangement, immune synaptic formation, and glycolytic capacity^{80,81}.

Notch and Hh signals play a synergistic role in the cellular development of multiple systems such as the digestive system, nervous system, and immune system^{82–84}. Notch signaling regulates Hh⁸⁵, and Hh signaling can directly or indirectly control Notch signaling through downstream activity⁸⁶. Regulation of the Notch pathway by Hh includes the following aspects: downstream effectors of Hh control the transcription of ligands and regulators of the Notch pathway, such as marginal proteins. The Gli protein exerts immediate and Notch-independent transcriptional regulation on certain Notch target genes^{87,88}. Hh is controlled by Notch signaling in the following ways: The downstream effectors of Notch control the transport of Hh components (e.g., Ptch and Smo) to PC⁸⁹. The downstream effector of Notch controls Gli levels independent of transcription. Notch intracellular domain (NICD) plays a direct role in the transcriptional regulation of Gli genes⁹⁰. Furthermore, both Notch and Hh signals are inhibited by NUMB⁸⁷, which helps Notch1 to interact directly with the WW domain of itchy E3 ubiquitin ligase (ITCH) through ubiquitination and degradation of its polypyrimidine beam-binding protein (PTB) domain⁹¹. Similarly, NUMB directly inhibits the hedgehog pathway, while ITCH promotes the ubiquitination of Glis1⁹².

Conclusion

In this study, the genetic information of AD was analyzed using life science and computer analysis methods. The six key screened genes were verified in clinical samples. However, there was a limitation to the clinical sample size of this study. Moreover, the regulatory roles of the NF-kappa B signaling pathway, hedgehog-signaling, and notch-signaling in the AD continuum were not further elucidated. We plan to improve the research design in future studies and explore the disease mechanisms and potential intervention targets in greater depth.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available because they protect patient privacy, but are available from the corresponding author upon reasonable request.

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Author contributions

NS: Original draft. HYW: Project administration, Visualization. KS: Project administration, Formal analysis. YZ: Visualization. ZYZ: Formal analysis. JWG: Methodology. DHW: Methodology, Supervision. YHW: Writing—review & editing, Funding acquisition.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The study was conducted in accordance with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Zaozhuang Hospital of Traditional Chinese Medicine (2025-syyws-001). All enrolled subjects signed an informed consent form (Date:2025/02/27/ version.2.0).

Consent for publication

Written informed consent was obtained from the clinical participants.

Additional information

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