COMPREHENSIVE MULTI-OMICS STUDY TO EXPLORE THE DYSFUNCTIONAL MECHANISM OF ALZHEIMER'S DISEASE

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ABSTRACT

Background: *With the aging of society, Alzheimer's disease (AD) has become a severe problem. Its pathogenesis is complex and overlaps with healthy aging, and it cannot be conclusively diagnosed and treated.*

Materials and methods: *This study comprehensively analyzed its dysfunctional mechanisms through multi-omics. In the GEO database, we collected microarray data from 9 hippocampal gene samples from the control group and 22 hippocampal gene samples from the diseased group and collected whole-genome DNA methylation data.*

Result: *The dysfunctional mechanisms were explored by differential genetic analysis, co-expression analysis, enrichment analysis, regulator prediction, and methylation analysis. Three sets of differential gene analysis yielded 164, 1715 and 2689 differential genes, respectively. Three thousand seven hundred eighty-six differential genes were obtained by combining the three groups of differential genes and de-duplicating them ($P<0.05$). Five functional barrier modules were obtained by weighted gene co-expression network analysis. The critical genes for each module are SUB1, CYP2C9, MZT2B, SV2A, and CYP11B2.*

Conclusion: *The results of the enrichment analysis show that the dysfunctional module is mainly related to cell-cell signaling by wnt and Wnt signaling pathway. Through the pivotal analysis of TF and ncRNA, we predicted the regulators of the dysfunctional module and obtained key regulators (SP1, SHOX2, miR-519-5p, miR-520e, miR-320d). Methylation analysis revealed that there were 162 methylated modified genes, but the degree of methylation was relatively low. Based on the analysis of multi-omics, we believe that the dysfunctional mechanism of AD is due to the regulation of Wnt channels by genes (SP1, SHOX2), which leads to pathological changes.*

Keywords: *Alzheimer's disease, multi-omics, methylation, co-expression analysis, WGCNA, enrichment analysis, the regulator.*

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Introduction

AD has become a major social problem due to the aging of the global population and the lack of effective treatments. According to statistics, there are currently more than 40 million people in the world who have dementia. In the United States, its incidence is about 66 seconds/case. It is estimated that by 2050, a new case is expected to occur every 33 seconds, with nearly 1 million new cases each year[(1)]. Reports in France in 2016 showed that more than 850,000 people have AD and related diseases[(2)].

The cost of care for 35 million people with dementia exceeds $600 billion annually, accounting for about 1% of global GDP[(3)]. Pathologically, the pathological features of AD are the deposition of amyloid plaques and phosphorylated tau. Neuroinflammation has also been considered to be a pathology associated with Alzheimer's disease. Chronic viral, bacterial and fungal infections are causative factors of the inflammatory pathway in Alzheimer's disease[(4, 5)]. In AD, genome-wide association studies (GWAS), next-generation sequencing technology (NGS), linkage analysis, and candidate gene research help us map genetic

information. This will not only provide us with new insights into the pathogenesis of AD but also facilitate early targeted interventions in AD[6].

At present, the widespread adoption of genome-wide association studies provides compelling evidence. It is proved that the pathogenesis of AD is associated with genes related to various physiological functions[7]. In the amyloid cascade hypothesis, we hypothesized that neurodegeneration in AD is caused by abnormal accumulation of amyloid beta (Aβ) plaques. This hypothesis has been supported over the past two decades, and research in several therapeutic areas currently provides an effective treatment for the treatment of devastating diseases[8]. Targeting gene sequencing plays an essential role in Alzheimer's disease. For treatment, we used acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor agonists[9]. Studies have shown a close relationship between Alzheimer's disease and aging genes. We believe that interventions that slow down aging and promote healthy longevity can help alleviate the exacerbation of Alzheimer's disease[10].

In addition to DNA research, we have recently studied DNA methylation in Alzheimer's disease. DNA methylation is the most in-depth study of epigenetic regulation. The study of DNA methylation is epigenetic, while epigenetics represents the inheritance of phenotypic changes, independent of altered DNA sequences, and the process of epigenetic formation under pathological and physiological conditions has become clear[11]. Studies have shown that part of the differentially methylated region (DMR) associated with Alzheimer's disease, which is highly methylated, is independently associated with aging. It indicates that DMRs are rich in brain-specific histone features and binding motifs of transcription factors and play a role in AD[12].

Studies have determined that precipitation in the brain, beta-amyloid (Aβ) and phosphorylated tau protein lead to the formation of senile plaques, and DNA methylation plays a genetic role. Apoptosis, neuronal inflammation, oxidative stress, and mitochondrial dysfunction induce aggregation of Aβ leading to pathological progression of AD. DNA methylation is a reversible process that makes it the most promising target for the treatment of AD[13, 14]. The purpose of this study is to study the molecular mechanisms in the development of Alzheimer's disease through multi-omics, to establish a theoretical basis for clinical treatment and to accelerate the prevention and treatment of Alzheimer's disease, and to provide a reference for potential target genes.

Materials and methods

Data resource

All data were from GEO, the selected database number was GSE1297 microarray data, and the probe data in the experiment was GPL96. Gene Expression Omnibus is a high-throughput microarray submitted by the research community, as well as an international public repository of its next-generation sequence functional genomic dataset. This resource supports archiving raw data, which in turn allows us to process data[15]. From the hippocampus genes of 9 control group hippocampal genes and 22 Alzheimer's disease patients, we downloaded transcriptome microarray data from GEO for differential gene analysis and co-expression analysis[16].

Difference analysis

According to the microarray data of the GEO sample, we use R language for analysis and processing. In this study, the limma package of R language was mainly used to construct the expression data of differential genes, including preliminary screening and correction of data[17-19]. We used the Correct background function to calibrate and normalize the data, with a threshold of $P<0.05$. We used the quantile normalization method in the normalize Between Arrays function to filter out the control probes in the GPL96 data as well as the low-expressed probes.

We finally used the eBayes and lmFit functions to identify the differentially expressed genes in the dataset, using default parameters. In the three periods, 164, 1715 and 2689 differential genes were obtained, and then we combined their differential bases to obtain 3786 differential genes. We believe that it is an expression disorder molecule of AD.

Co-expression analysis

High-throughput biotechnology is now widely used in biology and medicine, enabling scientists to monitor thousands of parameters simultaneously in a specific sample. However, useful mining information from high-throughput data remains a considerable challenge. Weighted gene co-expression network analysis tools can detect clusters of highly related genes[20]. To explore the molecular mechanisms of gene expression during progression in Alzheimer's disease, we performed a differential analysis between samples from different periods of Alzheimer's disease and normal samples. Based on the obtained 3786 differential genes, we completed the expression profile of the differential genes. We used a weight-

ed gene expression network analysis (WGCNA)[21] to analyze the differential expression profile matrix obtained from the study.

It is mainly to explore the case of the synergistic expression of these differential genes. We use the correlation coefficient weighting method to take the N-th power of the correlation coefficient between genes, and then get the correlation coefficient between any two genes (Person Coefficient). Before the results of the correlation coefficients are clustered, the links between the genes in the network need to be subject to the scale-free network distribution to be more biologically significant. After obtaining the weighted correlation coefficients, we cluster them and construct the clustering tree through the correlation coefficients between different genes. The different branches of the clustering tree represent different functional obstacle modules, and different colors represent different colors.

Different modules. Based on the ability of the gene to regulate in each dysfunction module, we unearth the essential genes that cause the dysfunction of the functional modules. In this study, five functional disorder modules were obtained, and five core genes, SUB1, CYP2C9, MZT2B, SV2A, and CYP11B2, were also obtained. We believe that it is related to the molecular regulation mechanism in the development of Alzheimer's disease.

Enrichment analysis

After obtaining the functional disorder module, it is necessary to understand the functions and signaling pathways involved in the critical genes of the module, in order to better understand the molecular mechanism of development in Alzheimer's disease. We performed an enrichment analysis of the functions and pathways of the genes of the dysfunctional module, which effectively explored the potential mechanism of gene expression in the pathogenesis of Alzheimer's disease.

Through co-expression analysis, we obtained five functional barrier modules. In the R language, we used the Clustering package of Bioconductor[22] for enrichment analysis of functions and pathways. For gene and gene clusters, we can perform statistical analysis and visualization of functional clustering through the Cluster profile package. we enrich the function and path of each module and build the corresponding function and access network.

Furthermore, we identify modules that participate in the proportion of corresponding functions and pathways to find the highest channel. The highest function of Count was found to be purine ribonucleotide metabolic process, and the highest path of Count was Huntington disease. The results of the enrichment analysis also show that it is mainly related to myelin, so we believe that myelin has a potential role in Alzheimer's disease.

Regulator analysis

Gene transcription and post-transcription are often regulated by non-coding RNA (ncRNA) and transcription factors (TF). We believe that ncRNA and TF are regulators of the entire molecular mechanism and that analysis of the regulators can help us better understand the molecular mechanisms in Alzheimer's disease.

We use bioinformatics to scientifically predict and test the role of the dysfunction module of Alzheimer's disease, thereby improving the accuracy and efficiency of the experiment.

In the process of ncRNA pivot analysis, we screened the control connections between each regulator and each module to be greater than or equal to 3, and the significance of the enrichment target between each module is p-Value<0.05. In the course of the TF pivot analysis, based on the hypergeometric test, we calculated the target significance of the enrichment in each module (p-value<0.05). The data is finally entered into Cytoscape to make a pivot-module network diagram.

Methylation analysis

The methylation chip data is downloaded from GEO, and the data is normalized by Rstudio so that the median value of the data is at the same level. The quality control (QC) is performed, and the Density-Bean diagram is obtained.

After culling samples with significant differences in variation, we performed SVD (singular value decomposition) to detect the significance of the variant components.

After the data was normalized, the methylation sites were analyzed for differences, and the package was used to find differential methylation sites, and differential methylation regions, and then the functions were interpreted, and the missing values were complemented. Differential genes and methylated modified Target genes were combined to obtain an intersection.

Based on the methylation modification level and differential expression of these differential genes, we identified the critical regulators modified by methylation.

Results

Determining expression disorder molecules in Alzheimer's disease

Alzheimer's disease is a common disease in the elderly. In three periods (1. Incipient; 2. Moderate; 3. Severe), differential genes in Alzheimer's disease were analyzed by comparing transcriptome data of 9 normal samples and 22 hippocampal samples.

The broad threshold P<0.05 was set, and 164, 1715, and 2689 differential genes were obtained in the three periods (Tables 1). Then we combined their differential bases and obtained 3786 differential genes (Table 1). Therefore, these 3786 differential genes are also considered to be expression disorder molecules in Alzheimer's disease, preparing for weighted analysis.

Color	Hudgens	Module
Blue	CYP2C9	m2
Brown	MZT2B	m3
Green	CYP11B2	m5
Turquoise	SUB1	m1
Yellow	SV2A	m4

Table 1: Hub gene of modules.

Identify relevant functional disorder modules in Alzheimer's disease

The Weighted Gene Co-Expression Network Analysis (WGCNA) tool can detect clusters of highly related genes, helping us to characterize the underlying pathogenesis of the biological disease. The weighted analyzed genes are clustered to form a module, and each module characterizes a potential mechanism of action. Each module also contains a core gene, and a core gene disorder can cause an abnormality in global function, leading to disease. In order to study the functional disorder module associated with progression in Alzheimer's disease, we need to construct an expression profile matrix in the sample of 3786 differential genes and their interaction genes. Then based on the weighted gene co-expression network analysis (WGCNA), we observed that these genes exhibited significant grouping in the sample. Genes with similar behavioral expression form a module for co-expression clustering.

In this study, we aggregated gene expression behavior into modules that help us observe the complex collaborative relationships between these genes from the perspective of expression behavior. We obtained five functional disorder modules (Fig. 1A, B), and further identified the essential genes of each module based on the functional disorder module, and obtained the five core genes of SUB1, CYP2C9, MZT2B, SV2A, and CYP11B2 (Table 1).

We associate the module with the phenotype data and plot it (Figure 1C). The MEblue module is associated with gene expression in Alzheimer's disease in a Severe period.

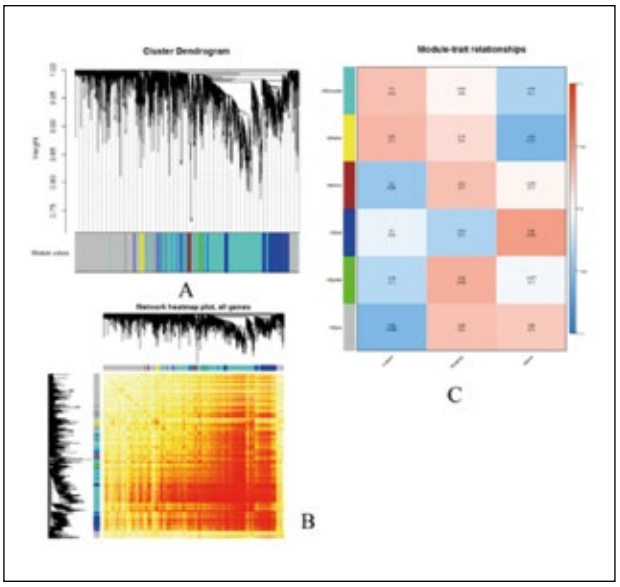

Figure 1: Synergistic expression of Alzheimer's disease. *A. The five co-expression groups obtained by clustering were identified as modules, and the five colors represented five co-expression modules. B. Expression heat map of all genes in the sample, whose expression behavior is clustered into five co-expression modules. C. Each row represents a module, each column represents a phenotype, the color of each cell is mapped by the corresponding correlation coefficient, the value is from -1 to 1, the color transitions from blue to white, and then transitions to red.*

Functions and pathways involved in the gene of interest

In diseases, functions and pathways are important mediators of physiological responses. We performed functional and pathway enrichment analysis on five functional barrier modules, resulting in a total of 17, 648 biological processes, 2246 cells, 3644 molecular functions, and 788 KEGG pathways (Table S2). The highest function of Count is purine ribonucleotide metabolic process, and the highest path of Count is Huntington disease. We extract some of the functions and pathways of interest from the table for analysis and bubble diagrams (Figure 2A, B). The results show that the enrichment is the myelin-related function and the pathway involved in endocytosis. In the figure, we can see that the circle of the myelin sheath is relatively large, indicating that its function is related to Alzheimer's disease so that we will focus on the biological function related to myelin. We enter the genes and their interactions into Cytoscape for

network analysis (Table S3). We use the median as a screening criterion. The higher the median, the more essential the gene is in this interaction network. From the table, we found STK24, COL5A1, KCNAB1, TAT, DCP2, and other large-medium genes, which represent these genes are indispensable in some interactions, and also an essential part of this network.

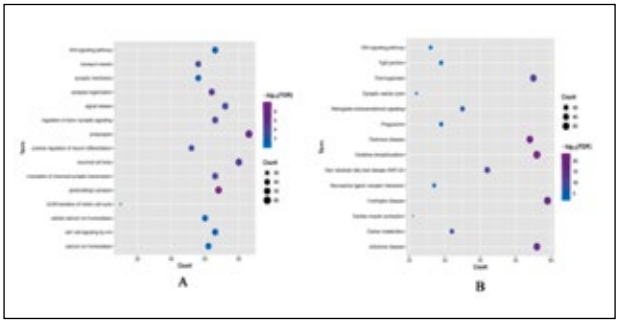

Figure 2: Functional and pathway enrichment analysis excerpts of the module gene.
A. Module gene GO function enrichment analysis excerpt. The color increases from blue to purple, and the enrichment increases significantly. The larger the circle, the more significant the proportion of the gene in the module that accounts for the GO function. B. Module gene KEGG pathway enrichment analysis excerpt. The color increases from blue to purple, and the enrichment increases significantly. The larger the circle, the more significant the proportion of the gene in the KEGG pathway entry.

ncRNA and TF that drive the development of Alzheimer's disease

Through the above analysis, we obtained the functions and essential genes related to the progression of Alzheimer's disease. However, from the perspective of systems biology and systems genetics, transcription and post-transcriptional regulation of genes have been considered as a critical regulator of disease occurrence and development, while ncRNA and TF are common regulators. Therefore, we performed a pivotal analysis based on the targeted regulation of the TF and ncRNA on the module gene and obtained a network diagram of the relationship between TF and ncRNA (Fig. 3A, B).

The results showed a total of 708 ncRNAs, and it involved 797 ncRNA-Module pairs with 48 TFs and 48 TF-Module pairs (Table S4, 5). In the ncRNA pivot analysis, the modules linked to ncRNA are miR-519-5p, miR-520e, miR-320d. In the TF pivot analysis, the module (SP1, SHOX2) is connected to the TF. These regulators show their association in the analysis of the functions and pathways in which our genes of interest are involved. Therefore, we believe that SP1, SHOX2, miR-519-5p, miR-520e, miR-320d regulators have potential regulatory effects in the progression of Alzheimer's disease.

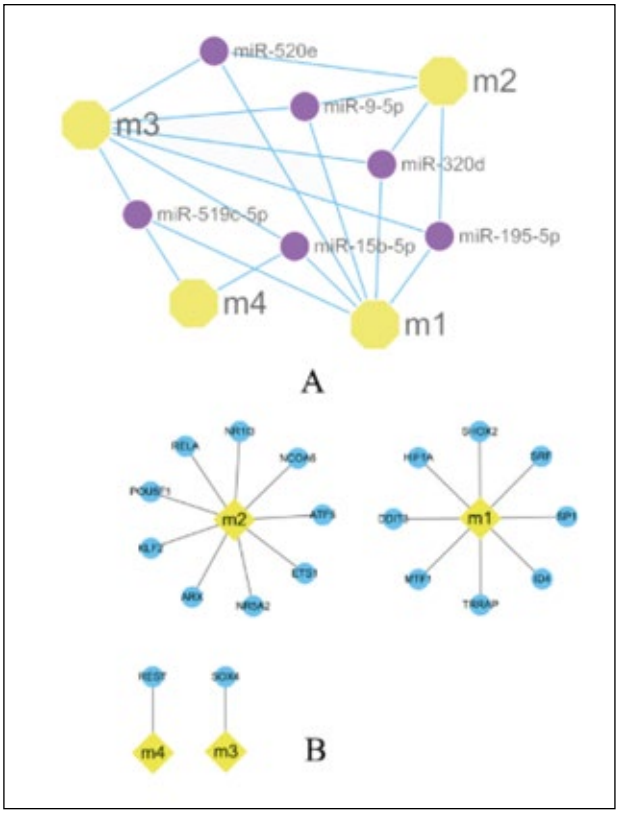

Figure 3: Modulatory effects of regulators on dysfunction modules.
A. The yellow octagon represents the module, and the purple circle represents the ncRNA. B. The yellow prism represents the module, and the blue circle represents the TF.

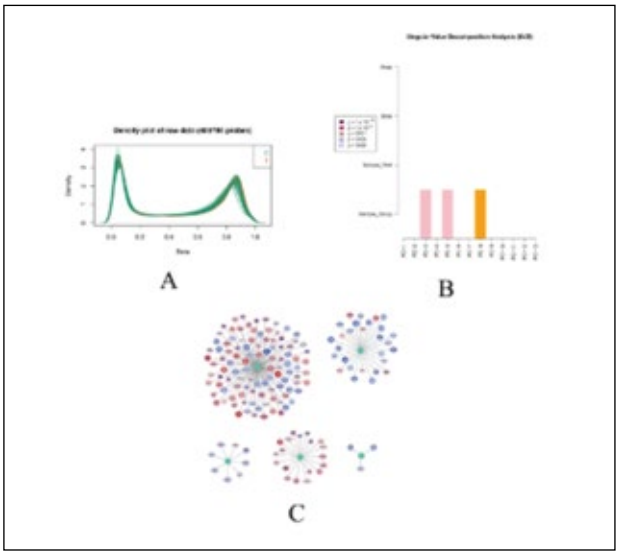

Figure 4: Methylation analysis of Alzheimer's disease.
A. Before methylation data is standardized, green represents a standard sample and red represents a disease sample. B. The main component of the difference before methylation data is normalized, and the color represents the magnitude of the P value. C. Levels of methylation modification of different genes. The blue-green circle represents the module, the nucleus of the node is the level of gene methylation, the edge of the node is the expression value of the differentially expressed gene, and the color of the node from blue to red represents the value from low to high.

Methylation analysis of Alzheimer's disease

DNA methylation mainly occurs in the process of disease and has multiple regulatory effects. We downloaded methylation chip data for Alzheimer's disease from GEO, including five control groups, 5 Braak I-II, 5 Braak III-IV, and 5 Braak V-VI samples. This study uses Rstudio to process the data, and we normalize the data and output the results (Figure 4A, B). From the figure, we can see that the outliers are removed before the standardization process, and some marked differences are avoided to cause the results to change.

We analyzed the difference components, and the results showed that the cause of the difference was biological treatment, not caused by technical factors, so no batch correction was needed. The normalized data can be used for measurement comparisons under various experimental conditions, eliminating non-experimental differences between measurements. We screened the value of q value<0.05 as the differential methylation site and then backtracked the target genes of the differential methylation site (Table S6), with a total of 4436 differential genes.

For the differential genes and the target genes backtracking the differential methylation sites, we combined them to obtain an intersection and identified 162 methylated modified differential genes. Mapping was based on the level of methylation modification and differential expression of the differential genes, and we identified vital regulators that were modified by methylation (Fig. 4C, Table S7).

Discussion

Alzheimer's disease is a common disease in modern medicine. The pathological features of AD are a synaptic loss (mainly in the neocortex) and deposition of certain unique lesions (results of protein misfolding) throughout the brain.

Sedimentation is mainly divided into:

• Age spots are composed of amyloid (Aβ) core and neuritis components;

• Neurofibrillary tangles are mainly composed of hyperphosphorylated tau;

• Cerebral amyloid angiopathy is a microvascular disease that affects the capillaries and arterioles of the cerebral cortex[23].

The amyloid cascade hypothesis is currently interpreted to explain the pathogenesis of Alzheimer's disease. The "amyloid cascade hypothesis" does not fully explain neuronal damage in AD, as evidenced by autopsy and imaging studies[24]. Scholars believe that neuroinflammation plays an important role in neurodegenerative diseases. This study collected microarray data (GSE1297) from the transcriptome of 31 subjects, 9 of whom were in the control group and 22 in Alzheimer's disease. The data were obtained from the hippocampal gene[25], and we performed differential analysis and co-expression analysis. After analysis, a total of five related functional disorder modules were obtained, including the five core genes of SUB1, CYP2C9, MZT2B, SV2A, and CYP11B2. The MEblue module was associated with gene expression in Alzheimer's disease during a Severe period, and the others did not show a significant correlation. Therefore, we judge that the core gene is more prominent in the late stage, and the pathogenesis is also gradual. Based on bioinformatics identification algorithms, we quantify the amount of protein/metabolite in cells using high-throughput methods, which significantly accelerates the exploration of the function and dynamics of complex biological systems. WGCNA was developed and applied to high-throughput microarrays and RNA-Seq datasets. In the R language, WGCNA provides functions such as network construction, module detection, gene selection, topology attribute calculation, data simulation, visualization, and functions interfaced with external software[21, 26].

In this study, we used the analysis method of WGCNA co-expression network to explore the obtained functional disorder module further and found that the highest function of Count is purine ribonucleotide metabolic process, and the highest path of Count is Huntington disease. The bubble map of the enrichment analysis shows that the functions associated with myelin and the pathways involved in endocytosis are relatively large. In the analysis of the interaction network, we found genes with larger mediators including STK24, COL5A1, KCNAB1, TAT, and DCP2. It represents the indispensability of these genes in certain interactions and is an important part of this network. In addition to WGCNA, DNA methylation analysis also plays a role in Alzheimer's disease. DNA methylation (5-methylcytosine, 5MC) identifies AD by altering several epigenetic markers[27]. Analysis of methylation was performed by methylation DNA immunoprecipitation-DNA microarray (MeDIP-chip). By quantifying differential methylation region analysis, we can identify differentially methylated regions (DMRs) and select differential methylation genes (DMGs) carrying at least three DMRs for pathway analysis. In healthy elderly and AD patients, based on methylation studies of

blood and brain, we found that methylation of promoters (SORL1, ABCA7, HLA-DRB5, SLC24A4, BIN1, OPRD1, ANK1, TREM2) is a difference in expression[28-36].

Studies have also shown that the DNA methylation cycle helps regulate the BMAL1 rhythm in the brain, and down-regulation of the BER gene may be a useful biomarker for the disease[37, 38]. Using genome-wide DNA methylation to analyze samples from normal and Alzheimer's disease at different times, we standardized the data (GSE45775). All sample data were used for comparison in various measurements[33, 39, 40]. Most of the differential genes did not undergo significant methylation modification during the progression of Alzheimer's disease. Based on the above multi-omics research, we believe that the dysfunctional mechanism of AD may be the production of gene-regulated proteins (SP1, SHOX2, PM20D1, CYBA), which may cause inflammation and myelin changes, and eventually lead to pathological changes.

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