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A Multi-omics Atlas at Single-cell Resolution Revealing Key miRNA Regulatory Modules Driving Post-stroke Microglial Polarization

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Abstract: *Ischemic stroke (IS) is the most common form of stroke. It triggers complex neuroinflammatory responses involving diverse cell populations within the central nervous system. Among these, microglia exhibit significant functional heterogeneity in IS. MicroRNAs (miRNAs), which regulate gene expression at the post-transcriptional level, are increasingly recognized as key modulators of neuroinflammation and potential biomarkers for IS; however, their specific relationships with microglial subtypes remain poorly defined. This research obtained 29 miRNAs through comparing correlation analysis between mRNA and miRNA. Through manual annotations and microglial populations, microglial functions are grouped into 5 groups. The process obtained increasing amounts of M1-like pro-inflammatory cells and decreasing amounts of M2-like anti-inflammatory populations. Several miRNAs, including miR-21-5p, let-7e-5p, miR-149-5p, miR-615-3p, and miR-218-5p, were shared across both M1 and M2, suggesting central roles in regulating polarization balance. Biological processes linked M1-associated targets to immunity, whereas M2-associated genes were enriched for cellular division and differentiation. These findings highlight miRNA-mediated networks that may shape microglial responses and influence IS progression. These findings provide insight into the mechanisms underlying microglial heterogeneity and offer potential directions for developing diagnostic biomarkers and therapeutic targets for ischemic stroke.*

Keywords: *Microglia, Ischemic stroke, Single cell RNA sequencing, Transcriptome, Interaction Networks*

1. Introduction

Stroke is a cerebrovascular disease that happens when blood supply to the brain is interrupted or reduced, preventing nutrients and oxygen to reach to the brain, eventually resulting in death. Throughout the world 6.5 million people suffer from stroke and ischemic stroke (IS) accounts for 87% of all strokes, making it an important medical condition researched by scientists throughout the world [1]. IS was caused by a blood clot or a narrowed artery, disrupting blood supply to the brain. The IS condition happens at various extents. The only two effective ways of curing IS was thrombolysis and thrombectomy, which could only effectively resolve the condition in the short term. It would resolve the IS condition but would inflict a series of reperfusion injuries within the brain, such as inflammation and oxidative stress. These long term conditions caused by IS are still to be researched. However, scientists recently have found evidence demonstrating correlations between the neuroinflammation and microglia and RNA molecules. The understanding of the correlation networks between target genes and RNA would allow researchers to design therapeutics for the long term effects of IS[2].

Microglia are resident immune cells in the central nervous system (CNS). They act as key regulators of the neuroinflammatory responses. Evidence indicates that there is a strong correlation with these key regulators and secondary brain injury and tissue repair in IS[3]. Microglia have a variety of functions, displaying microglial heterogeneity, which has a major impact while discussing the IS condition. They cause different probabilities of having the condition through sex, exhibiting morphological changes, connecting with aging, regulating other cells and genes while also being regulated[4]. After injuries in the brain, microglia polarize M1 and M2 phenotypes. M1 microglia release inflammatory cytokines that would help fight infections but the over activation of these microglia worsen brain damage and hinder recovery. On the other hand, M2 microglia help to resolve inflammation and promote the healing and repair response. Encouraging a shift from M1 to M2 therefore demonstrates a promising therapeutic strategy for the recovery of IS[5]. The microglia function allows us to look more closely at the specific

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cytokines and genes they regulate and allow us to observe the connection between the two.

microRNA (miRNA) are small, non-coding RNA molecules that regulate gene expression post-transcriptionally and have the ability to target hundreds of mRNA and influence the expression of a variety of genes, controlling development, metabolism, apoptosis, differentiation, and cellular signaling[6][7]. Increasing research demonstrates a high miRNA activity within the brain, exhibiting implications for stroke. miRNA correlates closely with IS activity, regulating neuroinflammation, microglial polarization, cell death and survival, and modulating neuroregeneration and repair. miRNA have the ability of triggering functional alterations at all times of the stroke[8]. Single cell RNA sequencing (scRNA-seq) is a sequencing technique that analyzes the gene expression at the level of individual cells, producing raw data containing RNA sequences. scRNA-seq nowadays has become a common way of analyzing factors causing disease, specifically neurological diseases and cancer[9][10]. Increasing research implements scRNA-seq in the brain to identify specific cells that contains genes influencing neuroinflammation, often times identifying the brain cells of mice having middle cerebral artery occlusion (MCAO)[11].

Currently, the specific microRNA-mediated regulatory mechanisms of microglial heterogeneity remain unclear. Additionally, the relationship between microRNAs in human stroke patients and the microglial subpopulations identified at the single-cell resolution from experimental stroke models has not been systematically explored. To fill this gap, this study combines the mRNA/miRNA sequencing data from acute ischemic stroke patients with the single-cell transcriptome data of microglia from experimental stroke models. Through differential expression analysis, correlation modeling, and regulatory network reconstruction, this study aims to identify the microRNAs associated with different microglial subtypes and elucidate their potential roles in regulating the neuroinflammatory response. This integrated approach provides a framework for identifying candidate molecular regulators of microglial behavior and may offer insights into novel diagnostic biomarkers or therapeutic targets for ischemic stroke.

2. Materials and Methods

2.1. Data Source

This study collected two public datasets from the Gene Expression Omnibus (GEO) database. Dataset GSE202709 contains total mRNA and miRNA sequencing data for 6 patients with mild ischemic stroke, 6 patients with severe ischemic stroke, and 4 healthy control subjects[12]. Dataset GSE267240 contains single-cell RNA sequencing data for microglial cells from control mice and MCAO mice, covering both male and female samples, with a total of 4 samples[13].

2.2. Bulk mRNA and miRNA Data Processing

2.2.1. Quality Control

For mRNA data, quality control can help eliminate the influence of poor-quality data on subsequent analysis. This process uses FastQC (version 0.11.9) to generate base quality reports for each sequence, base content reports for each sequence, GC content reports for each sequence, N content reports for each sequence, and adapter content reports. Subsequently, an intuitive FastQC evaluation report is generated, making it easy to visually review all the contents.

For miRNA, after downloading the data and changing the file type, quality control has also been implemented. In order to process miRNA data, xargs (v4.8.0) is being used to evaluate the quality of the raw miRNA data. A visual FastQC evaluation report is created with similar report contents as mRNA.

2.2.2. Data Filtering

The process of removing the indicators in mRNA that do not meet the quality requirements is accomplished using the Trimmomatic (version 0.39) tool. For the data screening of miRNA, the two tools, fastq_quality_filter and fastx_trimmer, are employed, both of which are from the FASTX toolkit (version 0.0.14).

2.2.3. Sequence Assembly by Reference Genome Alignment

Obtain the reference genome file of H.sapiens (GRCh38) for the complete assembly and location determination of sequencing reads. This file can be obtained at the link https://genome-

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idx.s3.amazonaws.com/hisat/grch38_genome.tar.gz. During this process, mRNA data is processed using HISAT2 (v2.2.1), and miRNA data is processed using Bowtie 2 (v2.5.2).

2.2.4. Sequencing Read Count

The featureCounts (version 2.0.6) tool is used for counting and statistics of mRNA sequencing reads and miRNA sequencing reads, outputting the transcriptional expression levels under corresponding sample groups. The obtained expression matrix will be an important input file for subsequent differential expression analysis.

2.3. Upstream Analysis of scRNA-seq Data

After downloading scRNA datasets, Cell Ranger (v10.0.0) is used to process raw sequencing data and generate quantitative gene expression profiles. The tool Cell Ranger count process the data including alignment of sequencing reads to a reference genome, correction of unique molecular identifiers (UMIs) and cell barcodes to reduce technical errors, and accurate quantification of gene expression levels at the single-cell level.

2.4. Filter Low Quality Cells

Downstream analysis was performed using the Seurat (v5.3.1) R package. Cells with low gene counts, high gene counts, or a high proportion of mitochondrial genes were excluded to ensure data quality. The cell count depth was set within the range of 1,500-5,000, and the proportion of mitochondrial genes was ensured to be less than 10%.

2.5. Dimensionality Reduction and Cluster

The expression data was standardized and scaled through the "NormalizeData" and "ScaleData" functions, thereby identifying the highly variable genes (HVG). Then, principal component analysis (PCA) was used for dimensionality reduction. Subsequently, the first 12 principal components were utilized for cluster analysis, which was applied to the entire dataset. Finally, the clustering results were visualized at different resolutions using the UMAP method (with parameters set to 0.3).

2.6. Annotations on Microglial Populations

Regarding the specific functions of microglia in the brain, by reviewing and organizing the labeled genes of microglia representing each functional group (see Supplementary Materials), manual annotation was carried out. Subsequently, a DotPlot was created to observe the expression characteristics of the labeled genes in the clusters, and five functional groups of microglia were identified.

2.7. Calculate the Characteristic Expressed Genes between Cell Clusters

Use the "FindAllMarkers" function to determine the expression differences among different functions of microglia under the condition that the p-value is less than 0.05 and the absolute log2 Fold Change (log2FC) value is greater than 1. Based on these results, the top 30 genes with the highest Log2FC values were selected for further analysis.

2.8. Differential Expression Analysis

Consider the changes in gene expression differences between IS patients and normal individuals at different stages, and process the mRNA and miRNA expression matrices using the DESeq2 (v1.48.2) tool. Differentially expressed genes (DEGs) were identified using a threshold of $p < 0.05$ and $log2FC > 1$. Subsequently, the VennDiagram package (v1.7.3) was employed to determine the intersection of DEGs across different comparison groups.

2.9. Correlation Analysis

miRNAs that were significantly dysregulated in both mild and severe ischemic stroke were selected for the downstream analysis. Two different correlation analysis were being conducted: miRNA-mRNA and miRNA-scRNA. For both correlation analysis, the corr.test function was used to determine the correlation between the miRNA and mRNA and miRNA and scRNA. Pearson correlation analysis was

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conducted between the expression levels of the two RNAs in each correlation analysis to identify potential regulatory relationships. miRNA–mRNA pairs and miRNA-scRNA paris with significant correlations were preserved, and correlation heatmaps were constructed using ggplot2 (v4.0.1).

2.10. Predict miRNA Transcriptional Factors and Target Genes

Important miRNA were uploaded to TransmiR to acquire the transcriptional factors (TF) and select TFs with p-values less than 0.05 and fold greater than 1.5. Reference datasets from miRTarBase and TargetScan were downloaded and uploaded to Cytoscape. CyTargetLinker is then used to create networks demonstrating the correlation between these target genes and miRNA.

3. Results

3.1. Identification of Differentially Expressed miRNAs and mRNAs in IS

The results of the differential expression analysis revealed that compared with the normal group samples, 160 mRNAs showed significant differences in the acute mild state of IS (P value < 0.05, Figure 1A: group 1); 198 mRNAs showed significant differences in the acute severe state of IS (P value < 0.05, Figure 1A: group 2). When comparing the mild group with the severe group (Figure 1A: group 3), 33 mRNAs (p value < 0.05) showed differences. For the results of miRNA differential expression, 29 miRNAs showed significant changes in their expression levels at different stages of IS, which might indicate that they play an important role in the development process of IS (Figure 1B). Table 1 presents the specific results of these 29 miRNAs.

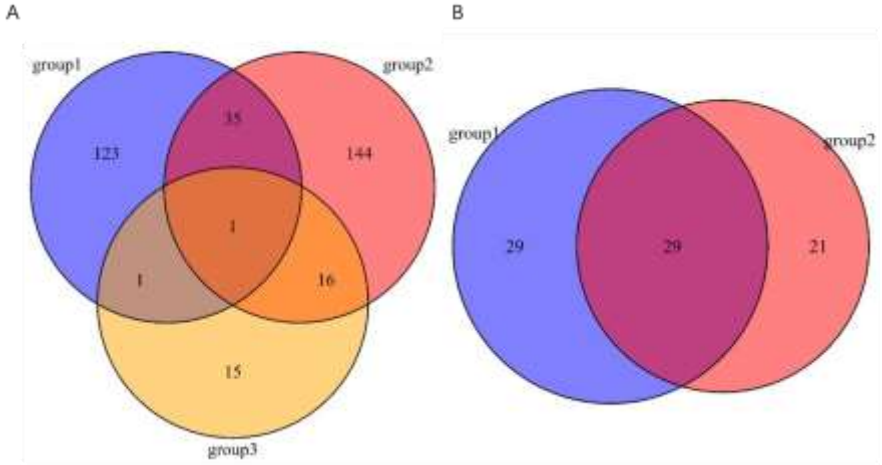

Figure 1: Venn diagram demonstrating amounts differentially expressed miRNAs and mRNAs. A: Differentially expressed mRNA in both normal, mild, and serious conditions for IS; B: Differentially expressed miRNA amounts in both IS mild group and IS serious group.

Table 1: Display of important miRNA that are correlated with IS.

Group Design	Presentation of Candidate miRNA results
IS mild group vs IS serious group	hsa-miR-21-5p, hsa-miR-224-5p, hsa-let-7e-5p, hsa-miR-30a-5p, hsa-miR-335-3p, hsa-miR-506-3p, hsa-miR-98-5p, hsa-miR-218-5p, hsa-miR-125b-1-3p, hsa-miR-615-3p, hsa-miR-30a-3p, hsa-miR-455-5p, hsa-miR-99b-3p, hsa-miR-129-5p, hsa-miR-532-3p, hsa-miR-10a-5p, hsa-miR-381-3p, hsa-miR-149-5p, hsa-miR-99b-5p, hsa-miR-6529-5p, hsa-miR-9-5p, hsa-miR-196a-5p, hsa-miR-6775-3p, hsa-miR-10400-5p, hsa-miR-452-5p, hsa-miR-146b-5p, hsa-miR-210-5p, hsa-miR-7-5p, hsa-miR-7155-3p

3.2. Construction of miRNA-mRNA Regulatory Networks Associated with IS Severity

To explore potential regulatory relationships, miRNAs that were significantly different for expression in both mild and severe ischemic stroke were selected for correlation analysis with differentially expressed mRNAs. Pearson correlation analysis identified numerous significant miRNA–mRNA pairs

ISSN 2618-1584 Vol. 8, Issue 3: 63-71, DOI: 10.25236/FMSR.2026.080309

between normal and mild groups but limited significant miRNA-mRNA pairs between normal and serious groups, indicating that the mild condition allows for more correlation between these pairs than the serious condition. Between mild and serious, most significant miRNA-mRNA pairs occur in up regulated pairs.

3.3. The Functional Involvement of Microglia in IS

Single-cell RNA sequencing data from control and MCAO mouse brains were analyzed to characterize microglial responses to ischemic injury. After quality control and normalization, unsupervised clustering identified distinct microglial subpopulations with unique transcriptional profiles.

After obtaining 13 clustering results (Figure 2A), the different functions of microglia were identified, and five microglial cell groups related to IS were determined: the process from the resting state to the polarized state, the induction of neurodegenerative diseases, the M2 type anti-inflammatory effect, the influence of gender differences, and the M1 type inflammatory effect. For MCAO patients, most microglia have the M1 type pro-inflammatory effect, are influenced by gender differences, and have the function of transitioning from the normal state to MCAO. The microglia with anti-inflammatory effects will have an impact during the transition from the normal state to MCAO.

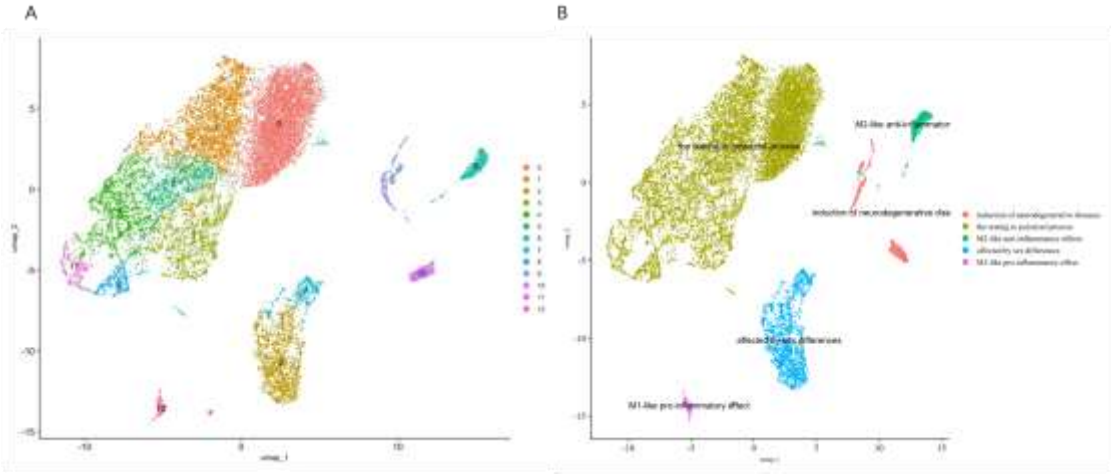

Figure 2: UMAP results for microglial cells in mice. A: Demonstrates the different clusters of microglial population; B: Demonstrates 5 different microglial functions.

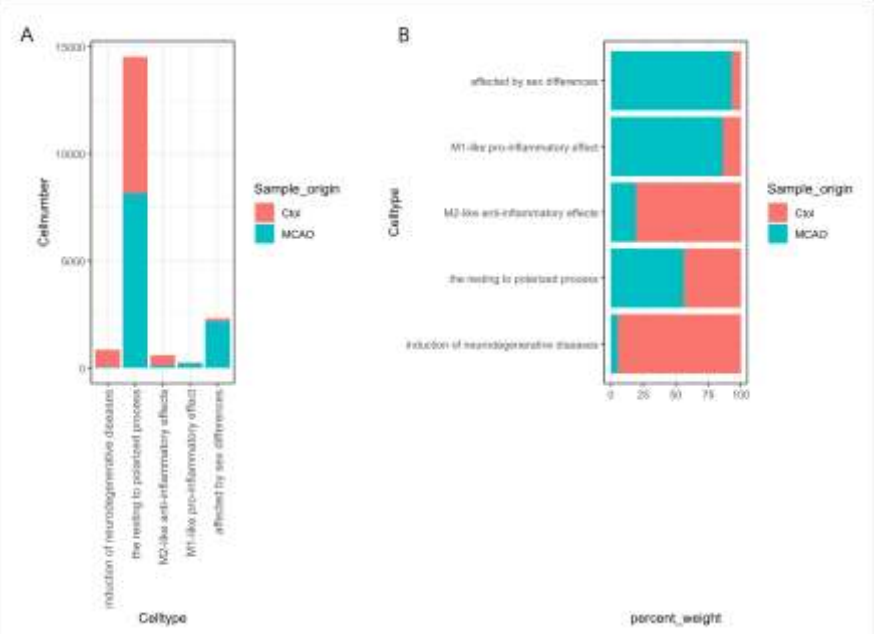

Figure 3: Frequency bar graphs displaying the cell number in different groups of microglial functions; A: Displays the total cell number; B: Displays the relative cell number in percentage.

ISSN 2618-1584 Vol. 8, Issue 3: 63-71, DOI: 10.25236/FMSR.2026.080309

The microglial cells with functions that are significnatly affected by the MCAO condition are determined through the comparison between the control cell and the condition cell. The study identified functions that were similar in both control cells and MCAO cells (the process from quiescence to polarization), as well as functions that were more abundant in control cells (inducing neurodegenerative diseases). Both of these indicate no significant difference after applying the MCAO conditions (Figure 3). The M1 and M2 functions were retained because, due to the influence of the MCAO conditions, more microglia cells would polarize into M1 inflammatory cells and would trigger a relatively smaller number of M2 anti-inflammatory cells. The influence of gender differences was also retained, as the proportion of MCAO cells was higher compared to the control cells, indicating a significant impact from the MCAO conditions.

3.4. Prediction Results of Key-miRNAs-Target Genes-Transcriptional Factors

Based on the above results, it was found that the cell numbers of these three functional microglial cell populations are affected by the IS state, namely: influenced by gender differences, M1 pro-inflammatory effect, and M2 anti-inflammatory effect. Based on the prediction results of target genes and transcription factors, a direct regulatory network of miRNAs and target genes was drawn.

In dysfunctional microglia influenced by gender differences, THBS1 is a potential target gene regulated by four key miRNAs (hsa-let-7e-5p, hsa-miR-381-3p, hsa-miR-30a-3p, and hsa-miR-21-5p). In the function of M1 type pro-inflammatory microglia, HLA-A and MMP9 act as potential target genes that may be regulated by several key miRNAs . HLA-A can be regulated by hsa-miR-9-5p, hsa-miR-615-3p, and hsa-miR-21-5-, while MMP9 can be regulated by hsa-miR-149-5p, hsa-miR-21-5p, hsa-let-7e-5p, and hsa-miR-9-5p. In the function of M2 type anti-inflammatory microglia, CCDC113 plays the role of a target gene and may be regulated by 4 key miRNAs: hsa-miR-149-5p, hsa-miR-7-5p, hsa-miR-455-5p, and hsa-let-7e-5p.

It is worth noting that hsa-miR-149-5p, hsa-miR-21-5p, hsa-let-7e-5p, hsa-miR-615-3p, and hsa-miR-218-5p all participated in both M1 and M2 cells. This highlights the relationship between the functions of M1 and M2 type microglial cells, as well as how they regulate similar functions under different polarization conditions.

3.5. The Functional Enrichment of Target Genes Regulated by M1/M2 miRNAs in Microglia

Based on the results of the 5 key miRNAs, all potential target genes regulated by these 5 miRNAs and having M1-type pro-inflammatory function and M2-type anti-inflammatory function were collected for functional enrichment analysis. The biological functions of the genes and the pathways they participated in were considered. Based on the minimum standard of p-value ($p < 0.01$), the GO entries and KEGG pathways participated by the target genes were screened.

Most of the top 10 biological processes with smallest p-values (p-values<0.01) in GO terms for target genes of M1-like pro-inflammatory effects relates to the chemotaxis, migration, and aggregation of immune cells . In the KEGG pathway, the IL-17 signaling pathway also indicates a similar biological process of immunity.

For M2-like anti-inflammatory effects, the top 10 biological processes with smallest p-values (p-values<0.01) in GO terms for target genes mostly relates to cellular division and differentiation (Figure 4).

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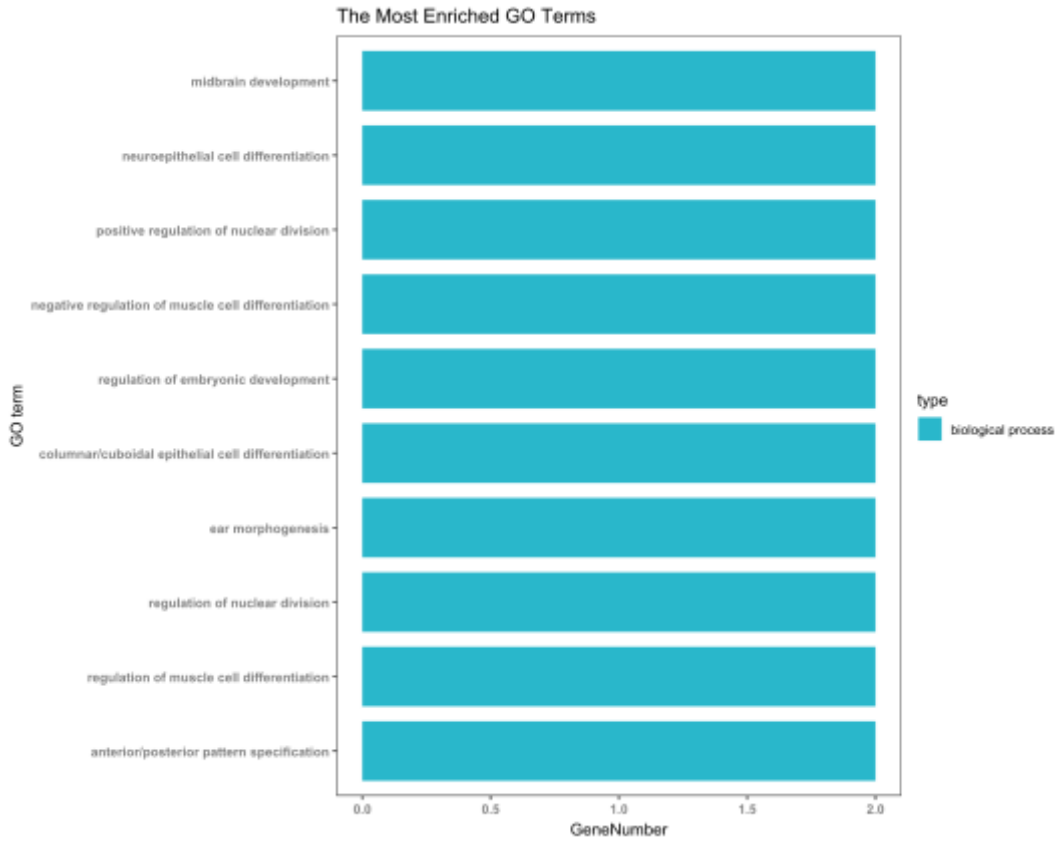

Figure 4: GO biological processes for M2-like anti-inflammatory effects (top 10 smallest p-values).

4. Discussion

This research discovered 5 key miRNAs that regulate target genes when dealing with the IS condition. Several miRNAs, including miR-21-5p, let-7e-5p, miR-149-5p, miR-615-3p, and miR-218-5p, appeared repeatedly in both M1 and M2 networks. Their recurrence across opposing functional states suggests that they may act as regulatory switches controlling polarization balance.

In terms of the M1-type pro-inflammatory function, the expression levels of miRNAs in the MCAO group decreased, leading to immune process disorder and disruption of the blood-brain barrier. Usually, reperfusion injury is accompanied by neuroinflammatory responses. Reperfusion injury exacerbates through damaging the vascular barrier, promoting the entry of peripheral immune cells into the brain, and aggravating ischemic injury. In this study, hsa-let-7e-5p was found to be associated with major inflammatory mediators (such as S100A8 and MMP9), highlighting its possible role in disrupting the blood-brain barrier. S100A8 is highly expressed after blood-brain barrier disruption and shows high levels of microglial cell polarization, indicating a connection between S100A8 and inflammation(C.-T. Jiang, et al, 2020). The main function of matrix metalloproteinase-9 (MMP9) is to degrade the vascular basement membrane or cell tight junctions, thereby causing disruption of the blood-brain barrier. On the other hand, the role of MMP9 in promoting inflammatory cell infiltration is also a widely studied topic in the field of cognitive decline(Yong, et al, 1998).

In M2-like anti-inflammatory functions, miRNAs regulate 4 different target genes----CCDC113, Otx2, MSX1, IGF2----that lead to biological processes of cellular division and differentiation. CCDC113 acts as an important gene when dealing with cellular development and growth in male fertility and sperm cells(Firat-Karalar, et al, 2014). However, in a study on post-stroke cognitive impairment rehabilitation training, CCDC113, as a gene that contributes to cognitive recovery processes, is also involved in processes related to synaptic remodeling, such as synapse guidance and neuroactive ligand-receptor interactions(Hong, et al, 2021). Otx2 is a transcriptional factor that determines the correct cellular composition of the midbrain region through regulating progenitor identity and neurogensis(Vernay, et al, 2005). OTX2 may also play a role in early morphogenesis of the central nervous system and is a key factor in pathways that help control proper neural development(Giudicelli, et al , 2001). In another

ISSN 2618-1584 Vol. 8, Issue 3: 63-71, DOI: 10.25236/FMSR.2026.080309

research, the choroid plexus in the human brain is responsible for secreting cerebrospinal fluid (CSF) and forming the blood-CSF barrier(Lun, et al, 2015). The MSX1-encoded Msh homeobox 1 transcription factor is involved in the early regulation of choroid plexus development and has a significant impact on the development of brain barriers(Muok, et al, 2023). If a gene with such functions becomes dysregulated in its expression, it could potentially lead to dysregulation in neuronal differentiation or function. Insulin-like growth factor 2 (IGF2) is a multifunctional factor expressed in the central nervous system and has been shown to be involved in physiological processes such as neurogenesis and immune homeostasis(Alberini, 2023). Similar evidence suggests that IGF2 can influence the activation state of microglia, helping cells shift toward an anti-inflammatory and repair-promoting phenotype(Guo, et al, 2023). The above evidence indicates that the downregulation of hsa-miR-615-3p may affect IGF2 expression, thereby mediating the polarization direction of microglia. Despite having different target genes for M1 and M2 microglial functions, they have the exact same miRNAs. As a result, if there is a change in expression levels (for example, significant down-regulation), it may cause different polarizations, leading to different microglial functions. Thus, causing different target genes to be expressed.

Nowadays, research on microRNAs (miRNAs) and ischemic stroke is increasing, aiming to improve treatment methods for this condition. In one study, Yang and others analyzed certain microRNAs associated with ischemic stroke. Through their research, they examined the relationship between microRNAs and infarct volume, identifying results for nine different microRNAs(Yang, et al, 2024). Li and others(S.-S. Li, et al, 2023) found that miR-212-5p, as a microglia-derived exosomal miRNA, has a neuroprotective effect. These trends demonstrate that miRNAs could be an important direction for the treatment of stroke or brain diseases in the future. This study aims to identify hsa-miR-149-5p, hsa-miR-21-5p, hsa-let-7e-5p, hsa-miR-615-3p, and hsa-miR-218-5p as subsequent miRNAs mediating the polarization effects between microglial M1 and M2 through the cross-regulation of transcriptional information and microglial cell functions under ischemic stroke, thereby indicating their potential in IS therapy.

5. Conclusion

Overall, this work identifies miRNA regulatory networks and highlights microglial polarization as a key driver of IS exacerbation. The recurrent miRNAs uncovered here represent promising biomarkers and potential targets for strategies aimed at restoring immune balance after cerebral ischemia.

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