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Identifying the biomarkers of multiple sclerosis based on non-coding RNA signature

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Abstract. – OBJECTIVE: miRNAs are key regulators in multiple sclerosis. To gain a better understanding of the molecular mechanisms of multiple sclerosis, differentially expressed microRNAs (DE-miRNAs) and genes (DEGs) were analyzed.

MATERIALS AND METHODS: The miRNA expression profile GSE43590 including 11 samples of peripheral blood T-cells from relapsing-remitting MS patients and 9 normal samples as well as gene expression profile GSE52139 including 8 periplaque samples and 8 normal samples were downloaded from Gene Expression Omnibus. Then, DE-miRNAs and DEGs were identified using limma. Moreover, the target genes of DE-miRNAs were screened. Additionally, the integrated regulatory network of DEGs, DE-miRNAs and targets was constructed using Cytoscape. What's more, the functional modules were also screened using MINE in Cytoscape. Lastly, the functional annotation of genes in modules was conducted using DAVID.

RESULTS: A total of 2394 DEGs were screened in 8 periplaque samples. Additionally, 296 DE-miRNAs were identified in the 11 samples of peripheral blood T-cells from relapsing-remitting MS patients. Besides, 6 functional modules (A-F) were screened. Among them, has-miR-197 could target HNF4A. What's more, HNF4A could interact with CYP3A4. Additionally, has-miR-125b could target ID1 and ID3. Besides, ID1 could interact with THBS1. Furthermore, functional enrichment showed that CYP3A4 was significantly related to vitamin metabolic process. For the pathway enrichment, ID1 and ID3 were significantly enriched in TGF-beta signaling pathway.

CONCLUSIONS: Some important DE-miRNAs (such as has-miR-197and has-miR-125b) might be crucial for MS by regulating the expressions of their target genes.

Key Words:
Multiple sclerosis, Differentially expressed miRNA, Differentially expressed gene, Regulatory network.

Introduction

Multiple sclerosis (MS), also referred as the disseminated sclerosis or encephalomyelitis disseminata, is a chronic autoimmune disease in central nervous system[1]. It has been found that the MS usually occurs between the ages of 20 and 50 with 3 main characters including the formation of lesions in central nervous system (also called plaques), inflammation and the destruction of myelin sheaths of neurons demyelination[2,3]. Especially, among the women of childbearing age, MS is very common which could bring risk when they requie the local anesthetics[4].

Recently, research has proven that periplaque demyelinated lesions was one of the hallmarks of MS[5] and many reports have illustrated the mechanisms related to MS. Study of Lieury et al[6] has demonstrated that the myelin-related, astrocyte-related immune-related genes were differentially expressed in the periplaque demyelinated lesions from cervical spinal cord of 8 patients with primary or secondary progressive MS. Additionally, it has been discovered that axonal damage mediated by T cell mediated always occurs concomitantly with demyelination in mice[7]. Besides, a high apolipoprotein E (APOE) 4 frequency and lower serum ApoE levels has been identified in mals with MS[8]. In other study[9], Sox17 has been identified to play a critical role in oligodendrocyte differentiation and periplaque demyelinated

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lesion repair. In addition, microRNAs has been demonstrated to be involved in MS. Former study[10] has proven that miR-326 could regulate TH-17 differentiation by targeting Ets-1 which is associated with the pathogenesis of multiple sclerosis. Besides, miRNA such as the dysregulations of miRNA-34a, miRNA-155 and miRNA-326 in multiple sclerosis lesions could reduce CD47 in brain resident cells, releasing macrophages from inhibitory control, thus, promoting phagocytosis of myelin[11]. However, the *in vivo* functions of most microRNAs are largely unknown.

Previous study[12] has found that CD4(+) $CD25^{high}$ regulatory T cells in peripheral blood was associated with MS. Additionally, Jernas et al[13] have identified differentially expressed genes (DEGs) and differentially expressed miRNAs (DE-miRNAs) for peripheral blood T-cells from relapsing-remitting MS patients according to the gene and miRNA microarray analysis. However, the important genes related to the incomplete demyelination in the cervical spinal cord of MS targeted by miRNAs were unclearly illustrated. In our study, the gene expression data from periplaque regions in multiple sclerosis spinal cords combined with non-coding RNA expression file of peripheral blood T-cells in multiple sclerosis were applied to screen the DEGs and DE-miRNAs, respectively. Then, the target genes of the screened DE-miRNAs related to the incomplete demyelination in the cervical spinal cord of MS were identified. Subsequently, miRNA regulatory network (including miRNA-target and target-DEG) and enrichment analysis were conducted to identify the potential biomarkers for MS.

Materials and Methods

Microarray Data

Expression profile of GSE52139[6] was downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) which was based on the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. A total of 16 samples are enrolled in the dataset including 8 periplaque samples from patients with MS and 8 normal appearing white matter samples from the same patients. In addition, the non-coding RNA expression file of GSE43590[13] was also downloaded from GEO including 11 samples of peripheral blood T-cells from relapsing-remitting MS patients and 9 normal samples from healthy individuals. This dataset was generated based on the platform of GPL14613 [miRNA-2_0] Affymetrix Multi-species miRNA-2_0 Array.

Differentially Expressed Genes and miRNAs Screening

The limma (linear models for microarray data) package in R/Bioconductor was used to screen the DEGs and DE-miRNAs[14]. The p-value < 0.05 and the |log2FC (fold change)| > 1 were used as the cut-off criteria.

Target Genes of DE-mi RNA

MiRTarBase database is a collection of experimentally validated microRNA-target interactions (http://miRTarBase.mbc.nctu.edu.tw/)[15]. Besides, TarBase 6.0 is a database gathering the exponential growth of miRNA targets with experimental support (http://www.microrna.gr/tarbase)[16]. For the miRecords database, it is an integrated resource of microRNA-target interactions (http://miRecords.umn.edu/miRecords)[17]. According to these three databases, the interactions between DE-miRNAs and their target genes (including the DEGs and the non-DEGs) were identified.

In addition, the biological general repository for interaction datasets (BioGRID: http//thebiogrid.org) is an open access archive of genetic and protein interactions that are curated from the primary biomedical literature for all major model organism species[18]. Human protein reference database (HPRD: http://www.hprd.org/) is a rich resource of experimentally proven features of human proteins[19]. Then, the interactions between the genes were discovered based on the BioGRID and HPRD.

Network Construction and Topological Properties Analysis

The network containing interactions between DE-miRNA-target (non-DEGs and DEGs) and target-DEGs was constructed and visualized using Cytoscape[20]. Subsequently, the topological properties analysis of the network was performed to analyze the differences between specific network (including the relationships of DE-miRNAs-targets, targets-DEGs and DE-miRNAs-DEGs) and the background network (constructed with the validated miRNAs and miRNA target gene) by plug-in network analysis in terms of degree, average shortest path length (ASPL), closeness centrality (CC), eccentricity (EC) and topological coefficient (TC).

Identification and Functional Annotation of Modules

The functional modules with significant differences and specificity related to MS were identified using the plug-in MINE of Cytoscape[21].

The online tool DAVID (The Database for Annotation, Visualization and Integrated Discovery)[22] was employed to perform the functional enrichment of EDGs in the modules based on the Gene Ontology (GO) database. A *p*-value less than 0.05 was used as the cut-off criterion for both the pathway and functional enrichment analysis.

Results

Differentially Expressed Genes and miRNAs Screening

A total of 2394 DEGs including 1625 up-regulated (such as *HNF4A*, *CYP3A4* and *THBS1*) and 769 (such as *ID3* and *FBXO11*) down-regulated DEGs were screened in 8 periplaque samples compared with the 8 normal samples. The top ten up-regulated and down-regulated DEGs are shown in Table I. Additionally, 296 DE-miRNAs, containing 295 up-regulated (such as hsa-miR-125b and hsa-miR-197) and 1 down-regulated miRNA (snoRNA: ENSG00000202498) were identified in the 11 samples of peripheral blood T-cells from relapsing-remitting MS patients compared with the 9 normal samples. The top ten up-regulated and the one down-regulated DEmiRNAs are shown in Table II.

Network Construction and Topological Properties Analysis

The specific network related to MS including the relationships of the DE-miRNA-target (non-DEGs and DEGs) and target (non-DEGs)-DEGs was constructed. Within the network, there contained 2368 strips and 626 nodes including 19 differentially expressed miRNAs, 121 target genes and 485 DEGs (Figure 1). Then, the background network was also constructed based on the miRNA-target databases of MiRTarBase, TarBase 6.0 and miRecords as well as the two protein-protein interaction databases of BioGRID and HPRD including 2809 nodes and 9023 strips. The five characteristic parameters including degree, ASPL, CC, EC and TC are shown in Table III. The ASPLs (miRNA = 3.40, gene = 3.20) in our network were shorter than those (miRNA = 3.96, gene = 3.90) of the background network. Moreover, the EC (miRNA = 5.68, gene = 5.34) were lower than those (miRNA = 7.00, gene = 7.05) of the background network.

Table I. The top ten up- and down-regulated DEGs in 8 periplaque samples compared with the 8 normal samples.

DEGs	ID	Gene symbol	*p* value	logFC
Up-regulated	1554142_at	*SUGT1P3*	8.32E-04	3.691891
	208273_at	*ZNF695*	3.52E-05	3.579989
	1552514_at	*WBP2NL*	3.38E-03	3.426142
	226475_at	*FAM118A*	7.96E-04	3.197906
	1559849_at	*ZNF605*	2.04E-04	3.128065
	232980_at	*LMBRD1*	1.02E-04	3.086673
	1565799_at	*RAB3IP*	5.14E-03	3.063671
	228776_at	*GJC1*	4.58E-02	2.943820
	231980_at	*DOK6*	2.26E-03	2.937933
	220978_at	*KRTAP1-3*	5.84E-04	2.878730
Down-regulated	219208_at	*FBXO11*	9.06E-04	-3.700820
	203634_s_at	*CPT1A*	5.28E-04	-3.616540
	219947_at	*CLEC4A*	3.88E-03	-3.406890
	218984_at	*PUS7*	1.33E-02	-3.335950
	221024_s_at	*SLC2A10*	8.03E-04	-3.331520
	230534_at	*ZNF678*	3.48E-02	-3.296350
	221003_s_at	*CAB39L*	5.95E-06	-3.067960
	222062_at	*IL27RA*	1.33E-02	-2.978010
	225219_at	*SMAD5*	7.79E-03	-2.973890
	211084_x_at	*PRKD3*	1.29E-03	-2.954860

DEGs: differentially expressed gene; FC: fold change.

Table II. The top ten up-regulated and the one down-regulated miRNAs in the 11 samples of peripheral blood T-cells from relapsing-remitting MS patients compared with the 9 normal samples.

DE-miRNAs	ID	*p* value	logFC	MiRNA symbols
Up-regulated	dre-miR-150_st	3.76E-05	3.141002	Has-miR-150
	spu-miR-92c_st	1.46E-04	3.032099	Has-miR-92c
	ptr-miR-30c_st	3.55E-04	2.990707	Has-miR-30c
	ppy-miR-494_st	6.21E-06	2.950469	Has-miR-494
	eca-miR-494_st	1.30E-06	2.946467	Has-miR-494
	ppy-miR-30c_st	1.53E-04	2.927614	Has-miR-30c
	v11_mmu-miR-197_st	6.27E-08	2.901760	Has-miR-197
	cfa-miR-494_st	2.93E-07	2.885010	Has-miR-494
	mne-miR-15b_st	9.18E-04	2.874558	Has-miR-15b
	ppa-miR-197_st	4.51E-08	2.873804	Has-miR-197
Down-regulated	ENSG00000202498_st	4.17E-02	-1.438550	snoRNA: ENSG00000202498

MS: multiple sclerosis; miRNA: microRNA; DE-mi-RNAs: differentially expressed microRNA; DEGs: differentially expressed genes; FC: fold change.

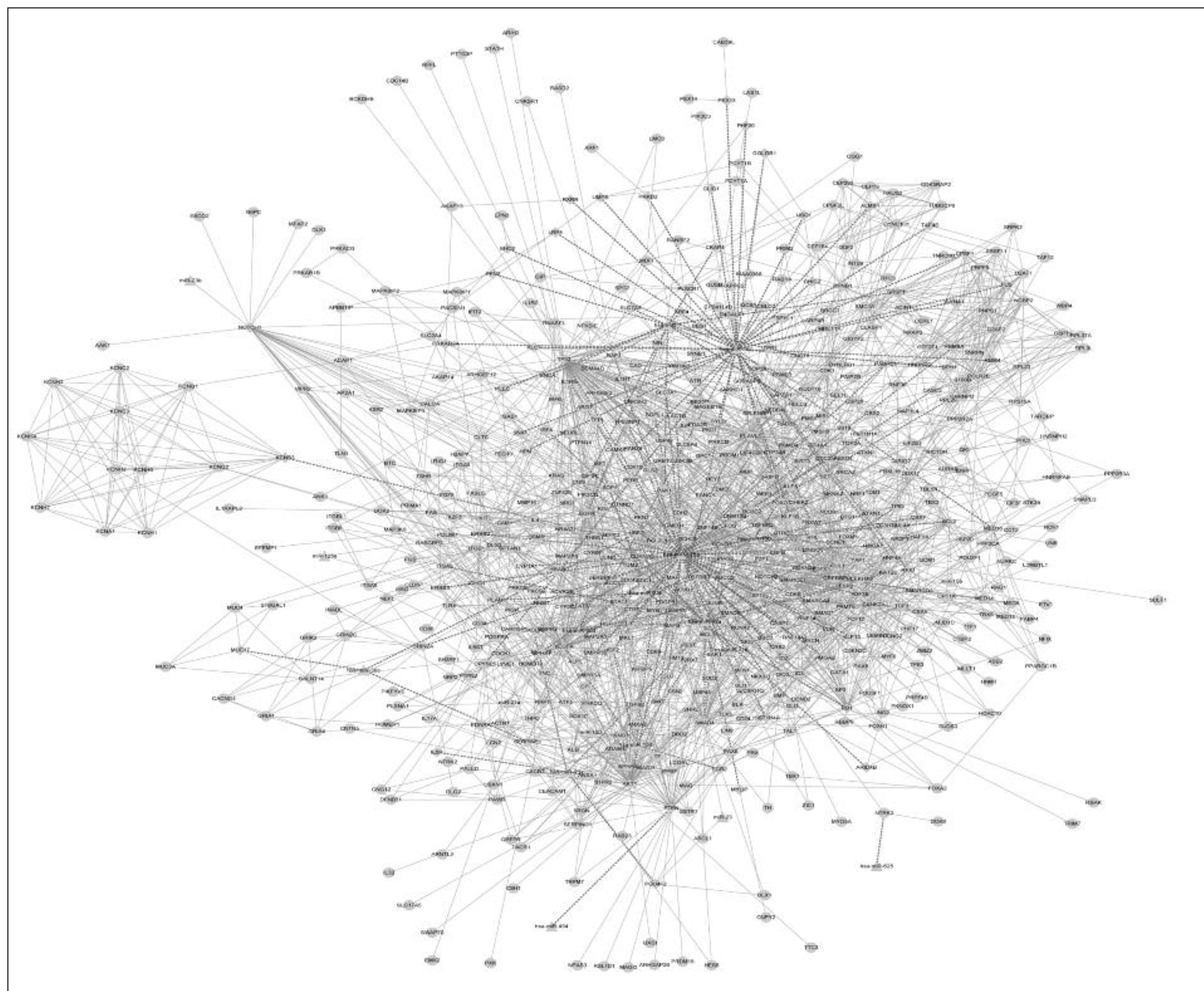

Figure 1. The integrated regulatory network including DE-miRNAs-target (DEGs and non-DEGs), DEGs-Targets (non-DEGs); the lines stand for the relation between genes; dotted lines stand for the relation between DE-miRNA and target; square nodes represent target genes; triangle nodes represent DE-miRNAs; cycle nodes represent DEGs.

Table III. The topological parameters of the specific and background network.

Network features	Specific network		Background network	
	MiRNA	Gene	MiRNA	Gene
Degree	8.30	7.50	11.37	5.57
ASPL	3.40	3.20	3.96	3.90
CC	0.32	0.30	0.29	0.27
EC	5.68	5.34	7.00	7.05
TC	0.21	0.25	0.18	0.30

miRNA: microRNA; ASPL: average shortest pathway length; CC: closeness centrality; EC: eccentricity; TC: topological coefficient.

Functional Modules Mining

Based on the relationship among specific network, 6 modules (A-F) including the DE-miRNAs, target genes and the DEGs were chosen for the further analysis. In module A, has-miR-197 could target *HNF4A*, and *HNF4A* could interact with *CYP3A4*. In module B, hsa-miR-125b could target *ID1* and *ID3*. Besides, *ID1* could interact with *THBS1* (Figure 2).

Modules Functional Annotation

According to the GO functional enrichment analysis, *CYP3A4* was significantly related to vitamin metabolic process (FDR = 0.001275699) in

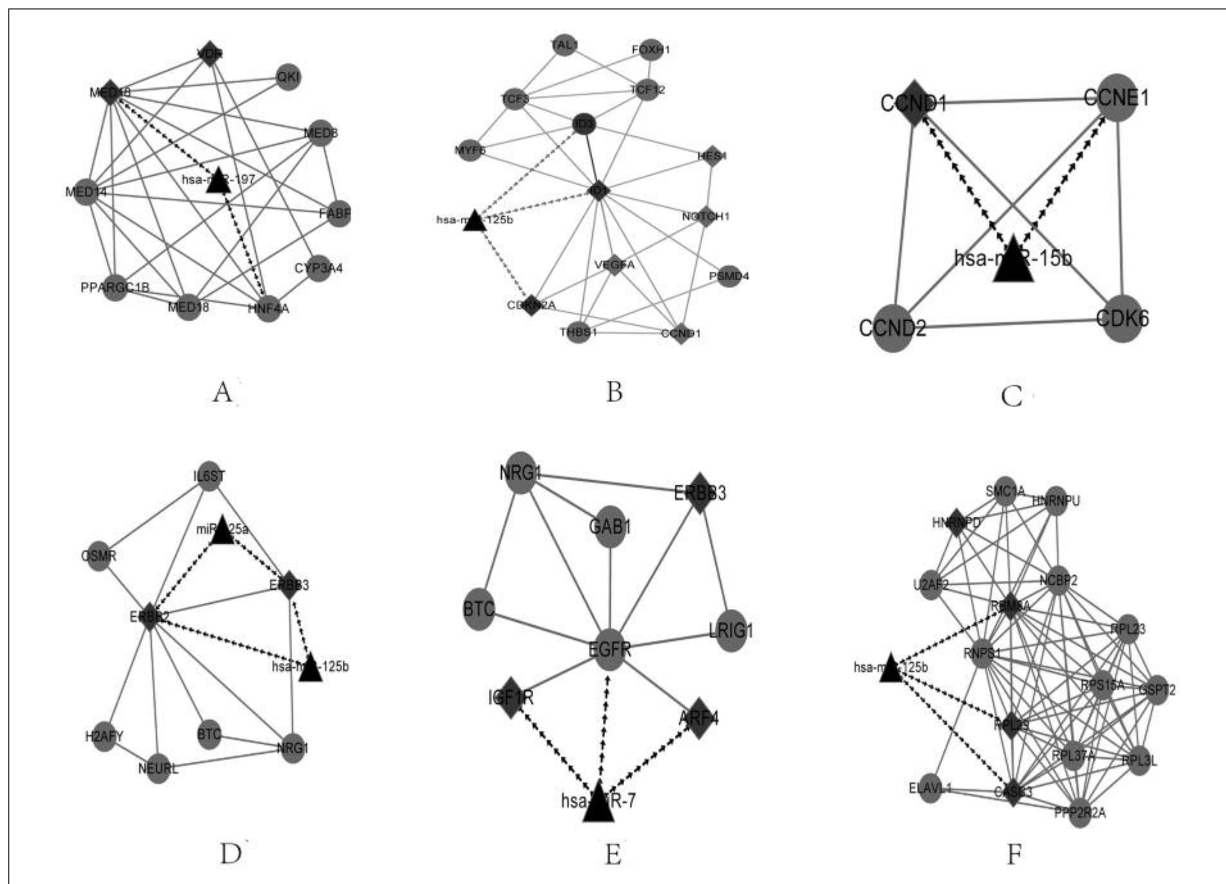

Figure 2. The 6 identified modules (A-F). The lines stand for the relation between genes; dotted line stand for the relation between DE-miRNA and target; diamond nodes represent target genes; triangle nodes represent DE-miRNAs; cycle nodes represent DEGs.

Table IV. The functional enrichments for the DEGs and DE-miRNAs in the modules.

Modules	DE-miRNAs	Terms	FDR	Gene symbols
Module A	hsa-miR-197	GO: 0006766~vitamin metabolic process	0.001276	CYP3A4
	hsa-miR-197	GO: 0001508~regulation of action potential	0.001931	QKI
	hsa-miR-197	GO: 0001816~cytokine production	0.002316	FABP4
Module B	hsa-miR-125b	GO: 0000165~MAPKKK cascade	0.001931	THBS1
	hsa-miR-125b	GO: 0000902~cell morphogenesis	0.002474	NOTCH1
	hsa-miR-125b	GO: 0006350~transcription	0.002474	TCF12
	hsa-miR-125b	GO: 0000080~G1 phase of mitotic cell cycle	0.003642	TCF3
Module C	hsa-miR-15b	GO: 0000082~G1/S transition of mitotic cell cycle	0.002147	CCNE1
	hsa-miR-15b	GO: 0000080~G1 phase of mitotic cell cycle	0.003603	CDK6
Module D	miR-125a(b)	GO: 0006323~DNA packaging	0.002463	H2AFY
	miR-125a(b)	GO: 0006323~DNA packaging	0.027949	H2AFY
	miR-125a(b)	GO: 0003074~regulation of diuresis	0.040735	BTC
	miR-125a(b)	GO: 0001817~regulation of cytokine production	0.080926	IL6ST

DE-miRNA: differentially expressed microRNA; DEG: differentially expressed genes; FDR: false discovery rate.

module A. Besides, *THBS1* was significantly enriched in MAPKKK cascade (FDR = 0.001930639) in module B (Table IV). As for the pathway enrichment, *ID1* and *IID3* were significantly enriched in TGF-beta signaling pathway (FDR = 0.038258228) (Table V).

Discussion

In this study, using bioinformatics methods, a total of 2394 DEGs including 1625 up-regulated and 769 down-regulated DEGs were screened out in 8 periplaque samples compared with the 8 normal samples. Additionally, 296 DE-miRNAs, containing 295 up-regulated and 1 down-regulated miRNAs were identified in 11 samples of peripheral blood T-cells from relapsing-remitting MS patients.

Using the DE-miRNAs-targets and the targets-DEGs, the specific network related to MS was constructed. Compared with the background network constructed using the all the miRNA-targets, gene-gene from the five databases, the ASPLs of miRNA and gene in our network were lower than those of the background network. Moreover, the EC of miRNA and gene were lower than those of the background network. All these showed that the network possessed higher correlation among the nodes than the background network. Therefore, the network constructed in our study was more effective.

According to the functional module mining, 6 important modules were identified. In module A, has-miR-197 could target *HNF4A*. Besides, *HNF4A* could interact with *CYP3A4*. Recently, it has been identified that *CYP3A4* was a human microsomal vitamin D 25-Hydroxylase which played a pivotal role in T cell homeostasis during

Table V. The pathway enrichments for the DEGs and DE-miRNAs in the modules.

Modules	DE-miRNAs	Terms	FDR	Gene symbols
Module A	hsa-miR-197	hsa00140: Steroid hormone biosynthesis	0.002147	*CYP3A4*
	hsa-miR-197	hsa03320: PPAR signaling pathway	0.038258	*FABP4*
Module B	hsa-miR-125b	hsa04115: p53 signaling pathway	0.002474	*THBS1*
	hsa-miR-125b	hsa04320: Dorso-ventral axis formation	0.003642	*NOTCH1*
	hsa-miR-125b	hsa04350: TGF-beta signaling pathway	0.038258	*ID1*
Module D	miR-125a(b)	hsa05322: Systemic lupus erythematosus	0.032849	*H2AFY*
	miR-125a(b)	hsa04012: ErbB signaling pathway	0.054720	*BTC*
Module E	hsa-miR-7	hsa04114: Oocyte meiosis	0.017547	*IGF1R*

DE-mi-RNA: differentially expressed microRNA; DEG: differentially expressed genes; FDR: false discovery rate.

the course of multiple sclerosis[23,24]. Additionally, the functional enrichment showed that *CYP3A4* was significantly related to vitamin metabolic process. It has been found that the lack of vitamin D could give rise to the surplus of effector T cells which were involved in autoimmune inflammations and, thus, conferring the MS[25,26]. Besides, polymorphisms in vitamin D metabolism related genes could lead to the multiple sclerosis[27]. Furthermore, vitamin D has a direct immunomodulatory effect on $CD8^+$ T cells of patients with early multiple sclerosis[28]. Accordingly, we speculated that *CYP3A4* indirectly targeted by has-miR-197 via *HNF4A* was involved in MS by the modulation of vitamin D metabolism.

In module B, hsa-miR-125b could target *ID3*. Moreover, *ID3* was significantly down-regulated. Former study has reported that the repression of the DNA-binding inhibitor *ID3* could limit the formation of memory $CD8^+$ T cells[29] which mediated severe autoimmunity and thus conferring the MS[30,31]. What's more, the functional enrichment displayed that *ID3* was significantly enriched in TGF-beta signaling pathway. Therefore, we predicted that *ID3* targeted by hsa-miR-125b could be involved in MS by the regulation of the $CD8^+$ T cells expression through the TGF-beta signaling pathway.

Additionally, we also identified that *ID1* targeted by hsa-miR-125b could interact with *THSB1*. Moreover, *ID1* was significantly enriched in TGF-beta signaling pathway. Previous report[32] has shown that TGF-beta signaling could regulate the differentiation of Th17. Moreover, Yang et al[33] have demonstrated that the deficiency of *THSB1* expression could lead to the reduction of Th17 differentiation and attenuates autoimmunity in mice. Additionally, the Th17 phenotype of $CD4^+$ T cell has been proven to mediate the autoimmunity leading to recurrent episodes of demyelination and axonal lesion in MS[34]. Consequently, we assumed that the interaction of *THSB1* and *ID3* targeted by hsa-miR-125b could be involved in MS by regulating the Th17 differentiated via TGF-beta signaling.

Conclusions

The identified DE-miRNAs of has-miR-197 and has-miR-125b in peripheral blood T-cells might participate in the pathogenesis of MS by regulating the expression levels of their target genes of *ID3* and *HNF4A*. However, further research is required to validate the results.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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