



An Integrated Bioinformatics Analysis of the Potential Regulatory Effects of miR-21 on T-cell Related Target Genes in Multiple Sclerosis

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Abstract

Background: Overexpression of miR-21 is a characteristic feature of patients with Multiple Sclerosis (MS) and is involved in gene regulation and the expression enhancement of pro-inflammatory factors including IFN γ and TNF- α following stimulation of T-cells *via* the T Cell Receptor (TCR). In this study, a novel integrated bioinformatics analysis was used to obtain a better understanding of the involvement of miR-21 in the development of MS, its protein biomarker signatures, RNA levels, and drug interactions through existing microarray and RNA-seq datasets of MS.

Methods: In order to obtain data on the Differentially Expressed Genes (DEGs) in patients with MS and normal controls, the GEO2R web tool was used to analyze the Gene Expression Omnibus (GEO) datasets, and then Protein-Protein Interaction (PPI) networks of co-expressed DEGs were designed using STRING. A molecular network of miRNA-genes and drugs based on differentially expressed genes was created for T-cells of MS patients to identify the targets of miR-21, that may act as important regulators and potential biomarkers for early diagnosis, prognosis and, potential therapeutic targets for MS.

Results: It found that seven genes (NRIP1, ARNT, KDM7A, S100A10, AK2, TGF β R2, and IL-6R) are regulated by drugs used in MS and miR-21. Finally, three overlapping genes (S100A10, NRIP1, KDM7A) were identified between miRNA-gene-drug network and nineteen genes as hub genes which can reflect the pathophysiology of MS.

Conclusion: Our findings suggest that miR-21 and MS-related drugs can act synergistically to regulate several genes in the existing datasets, and miR-21 inhibitors have the potential to be used in MS treatment.

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Keywords: Bioinformatics, MicroRNAs, Multiple sclerosis, T-cell

Introduction

Multiple Sclerosis (MS) is a common neurological disorder, which is more prevalent in women than men, and is identified by demyelination, chronic inflammation, and progressive neurological dysfunction^{1,2}. The etiology of this chronic inflammatory disorder is unclear; however, acute interstitial inflammation of nerves and the presence of multifocal sclerotic plaques in different parts of the peripheral and central nervous

system are common manifestations³. A fundamental characteristic of MS is an antigen-specific autoimmune response⁴. MS is a polygenic disease in which each gene has a small effect on the overall risk⁵. Recent genome-wide association studies have identified about 100 gene variants that are associated with a predisposition to MS. Most of these genes are considered to play a role in immunity⁶. MicroRNAs have been proposed

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as biomarkers for the early detection of MS ^{7,8}. Mature miRNAs are ~18–22 nucleotide single-stranded endogenous RNAs that bind to their target sequence on mRNA and regulate gene expression ⁹. miRNAs are responsible for regulating the expression of more than 60% of mammalian protein-coding genes ¹⁰. The expression profile of miRNA in MS patients has been studied and a large number of DEGs have been identified ¹¹. For example, there is strong evidence that miR-21 expression is up-regulated in MS patients compared with healthy controls ¹². These miRNAs are highly conserved non-coding RNAs involved in post-transcriptional regulation ¹³. miRNAs appear to be potentially useful as diagnostic biomarkers for MS, and it has been shown that the differential expression of these miRNAs is dependent on the time of onset and therapeutic stage. Recent studies have demonstrated that miRNAs may also have essential roles in MS pathogenesis ¹⁴. It is, therefore, possible that they could be used as both diagnostic markers and therapeutic targets in MS (Table 1) ^{15,16}. Although the function of miR-21 has been relatively well studied, its role in the development and progression of MS disease remains unclear. Satoh *et al* used proteomic profiling of MS brain lesions and analyzed the extracellular pathway to reveal the association between adhesion and integrin signaling in the progression of chronic MS lesions ¹⁷. Freiesleben *et al* assessed microarray data of peripheral blood and integrated genes of MS patients using a consensus method that determines the degree of agreement of inconsistent data ¹⁸. Studies performed using a variety of tissues such as brain lesions, and peripheral blood have been of relatively small cohort size and have not been replicated ¹⁸. It is worth pointing out that this study investigated microarray profiling of miRNA of appropriate size patient cohort, introduced the approach of the molecular network, and generated consensus interaction network between differentially expressed miRNAs and genes in T-cells of untreated MS patients to identify dysregulated miRNAs and their

target genes. To study the complex heterogeneity of multiple sclerosis for identifying MS-associated molecular functional networks in cells and dysregulated molecular mechanisms and pathways, integrative analyses seem to be more efficient in identifying a potential therapeutic target than the assessment of individual genes ^{6,19,20}. Bioinformatics analysis of gene expression profiling has recently been used to identify genetic alterations at RNA level, and transcription factors can be applied as biomarkers for human diseases such as MS ²¹. Bioinformatics analysis and systems biology can reveal molecular signatures comprising biomolecules at the protein level, drug, and RNA levels (miRNAs), and pathways have been used to obtain a more detailed understanding of the mechanisms involved in the pathogenesis of MS ^{22,23}. In the current study, a new integrated bioinformatics analysis was used to obtain a more detailed understanding of the mechanistic impact of miR-21 in MS, its protein biomarker signatures, RNA levels (mRNAs, miRNAs), and drug interactions by using the existing microarray databases of MS. MiR-21 was selected based on the reported dysregulation of this microRNA in MS ²⁴. Online databases such as HMDD v3.2, miR2Disease, and PhenomiR were used to determine the importance of miR-21 in gene regulation in MS. This study aimed to create a molecular network of miRNA genes and drugs, based on differentially expressed genes in T-cells of patients with MS, to identify the targets of miR-21, which act as important regulators and potential biomarkers in the early diagnosis, prognosis, and potential therapeutic targets for MS.

Materials and Methods

Data collection for gene expression analysis

Using a consistent specific platform, microarray datasets containing raw or normalized data were collected from the Gene Expression Omnibus (GEO) database. In order to collect comprehensive information, "multiple sclerosis", "Homo sapiens", and study type (Ex-

Table 1. An overview of the role of miR-21 in multiple sclerosis

Authors	Year	miR-21 function
Ma <i>et al</i> (25)	2014	- Up-regulated in peripheral blood mononuclear cells of relapsing-remitting MS patients - Expansion of Th1 and Th17 cells - Regulates cell apoptosis and growth factors
Lin <i>et al</i> (26)	2013	- Increases the synthesis of IFN- γ and IL-17A by T-cells and suppresses apoptosis via programmed cell death protein 4 (PDCD4) - Is responsible for sustaining the effector phase in effector T-cells
Piket <i>et al</i> (27)	2019	- Up-regulated during active MS disease
Tufekci <i>et al</i> (28)	2011	- Upregulated after the activation of TLR4, myeloid cells, and macrophage - Inhibition in the expression of IL12a, PTEN, and PDCD4 - Positive regulator of Foxp3 expression
Sheedy <i>et al</i> (29)	2015	- miR-21 in T-cell may also play an important role in self-tolerance regulation - Intrinsic miR-21 can also affect T-cell polarization
Fenoglio <i>et al</i> (30)	2011	- Significantly increased expression of miR-21 in relapsing-remitting (RR) MS patients - Activation of CD4+ lymphocytes
Muñoz-San Martín <i>et al</i> (12)	2019	- Overexpressed in the CSF of Gd+ and PBMCs of relapsing-remitting MS patients - Associated with clinical disability

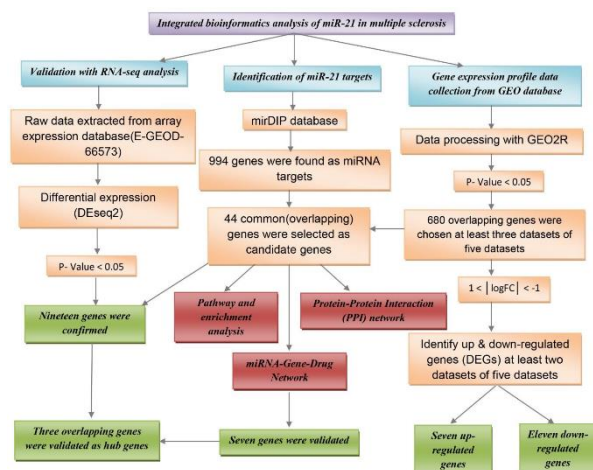


Figure 1. The bioinformatics flowchart used in the current study. DEGs: differentially expressed genes, PPI: protein-protein interaction, GEO: gene expression omnibus.

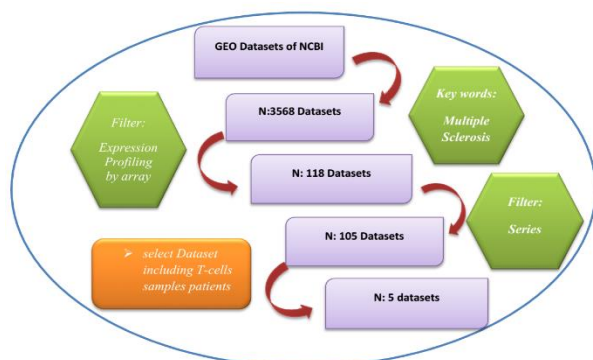


Figure 2. Outline of the protocol used for the search of multiple sclerosis microarray datasets from the GEO database.

pression profiling by array) were selected as keywords for the search in the GEO database. Finally, data were obtained from 5 mRNA microarrays (GSE43592, GSE13732, GSE16461, GSE78244, and GSE81279). The overall analysis process for this study is shown in figure 1 and the frame used for the selection of these datasets is shown in figure 2. The selected datasets included gene expression profiling using microarray in T-cells of patients with MS but datasets in which pa-

tients underwent treatment were excluded (Table 2). $p < 0.05$ was set to determine significant expression changes. The study was expanded by adding *in-silico* predicted miRNAs based on available MS-related genes and pathways.

Data preprocessing and analyzing of microarray

The GEO2R interactive web tool (<https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html>), using the GEO query and limma R packages, was applied for the analysis and comparison of the expression profiles of MS samples with controls in order to identify significant differences in gene expression after GEO2R analysis and obtain a final list of significant genes based on $p < 0.05$ (Cut off). The final results of the analysis of DEGs for up- and down-regulated genes were obtained by using cut off values for $p < 0.05$ and log Fold Change (logFC) > 1 or $\log FC < -1$. According to this novel approach of combining microarray analysis and bioinformatics tools, common differentially expressed genes were identified and selected between the predicted targets of miR-21 and microarray datasets using a Venn diagram for showing T-cells from patients with MS. To investigate the potential role of miR-21 in gene regulation in MS, publically available microarray datasets containing non-coding RNA of peripheral blood profiles of controls and patients were downloaded which corresponded to platform specifications of GEO database³¹. Studies in which patients were receiving therapy or in which samples were not obtained from blood, were excluded. At least seven replicates of the examined GSE31568 dataset containing each miRNA were measured, and the median of the replica was computed. To process the collected data more specifically, experimentally validated targets of miR-21 were searched and used to construct a primary miRNA-mRNA-drug regulatory network.

Prediction of miRNA target genes

The predicted targets of miR-21 were obtained from the online functional annotation tool, mirDIP 4.1 (<http://ophid.utoronto.ca/mirDIP/>), which provides 152 million human microRNA-target predictions, collected across 28 different resources (BCmicrO, BiTargeting, CoMeTa, Cupid, DIANA, EIMMo3, GenMir++, MicroRNA.org, miRBase, mirCoX, miRcode, miRDB, miRTar2GO, MAMI, MBStar, MirAncesTar, Mir-

Table 2. Characteristics of the five gene expression profiling datasets for multiple sclerosis in integrated bioinformatics analysis

GEO datasets	Data	Platform	Controls	MS patients	Tissue	Reference
MicroRNA						
GSE31568	Normalized	GPL9040	23	70	Peripheral blood cells	(31)
Genes						
GSE78244	Normalized	GPL17077	14	14	CD4+T cells	(32)
GSE13732	Raw	GPL570	37	28	CD4+T cells	(33)
GSE43591	Normalized	GPL570	10	10	T cells	(34)
GSE16461	Normalized	GPL1707	8	8	T cells	(35)
GSE81279	Raw	GPL21847	20	7	T cells	(36)

MAP, MirSNP, MirTar, Mirza-G, MultiMiTar, PAC-CMIT, PicTar, PITA, RepTar, RNA22, RNAhybrid, TargetRank, TargetScan, and TargetSpy)³⁷. Then, the target genes were aligned with the DEGs in MS, and this was used for further analysis.

Independent validation by RNA-sequencing (RNA-seq)

Independent validation of the 44 common genes as candidate key genes was derived by integrated microarray analysis results and miRNA targets and independent samples of MS and healthy controls from RNA-seq experiment (GEO accession no. of GSE 94266) were selected. The original experiment was designed to determine the Differentially Expressed Genes (DEGs) in MS patient versus healthy controls. Quality control of reads was analyzed using FastQC package (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Low quality reads and adaptor sequences were trim-med by the CLC Genomics Workbench 12.0.3 (QIAGEN, Germany). Mapping of short reads to the reference genome was performed using the CLC Genomics Workbench. Raw counts were obtained and used for Differential Expression (DE) analysis. The differential expression analysis was performed using DESeq2 and genes with $p \leq 0.05$ were defined as Differentially Expressed Genes (DEGs).

Functional and pathway enrichment analysis

The Gene Ontology (GO) enrichment analysis including Biological Process (BP), Molecular Function (MF), and cellular component (CC), and the Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses of common genes were carried out using the Enrichr database, which is a bioinformatics data platform consisting of an extensive biology knowledge database and analysis tools to align and explore significant biological information from large quantities of genes and protein collections³⁸. A $p < 0.05$ was used as the cut off criterion to determine the important pathways in which the genes are involved.

PPI network construction

The STRING (Search tool for the retrieval of interacting genes) database (<http://string-db.org/>) was used for constructing common DEGs network by calculating the protein-protein interaction.

Prediction of drug-gene interaction

Drugs and their target genes were downloaded from the drug-gene interaction database (DGIdb v3.0, www.dgldb.org)^{39,40}. DGIdb normalizes content from 30 different sources and provides access through an intuitive web user interface, Application Programming Interface (API), and public cloud-based server image⁴⁰. In addition, Cytoscape software was applied to extend gene-drug interaction network.

miRNA- mRNA-drug interaction network

mRNA-miRNA and drug-based disease-associated regulatory network were assessed by using microarray datasets in order to identify the relationship between

miR-21, differentially expressed genes, and well-known drugs in MS. To create networks between miRNA-genes and drugs, common genes between DEGs and predicted miR-21 targets and related drugs were selected to obtain the intersection for creating networks using Cytoscape software (<https://cytoscape.org/>).

Results

Verification of miR-21 in MS

In order to develop a miRNA gene-based disease-associated network, data were collected by three different methods to identify miRNAs associated with MS. MiR-21 was selected as a candidate biomarker in MS, based on previous findings regarding the role of miR-21 in gene regulation in the etiology of MS. There was a statistically significant increase in expression of miR-21 in the peripheral mononuclear cells of patients with Relapsing-Remitting (RR) MS compared to controls. For in silico analysis, the GSE31568 dataset contained 23 MS samples and 70 control samples and based on GPL9040 platform (febit Homo Sapiens miRBase 13.0), there was significantly up- and down-regulated miR-21 in peripheral blood cells (Table 3).

Identification of differentially expressed genes (DEGs) in MS patients

The five selected datasets were downloaded directly from GEO (<https://www.ncbi.nlm.nih.gov/geo/>) database and analyzed using GEO2R. They were identified as 7502, 14776, 1840, 3927, 140 DEGs in GSE43591, GSE13732, GSE16461, GSE78244, and GSE81279 and composed of up-and down-regulated expression based on criteria of log fold change > 1 or < -1 and $p < 0.05$ in MS as described in table 3 and figure 3. Genes of datasets that were differentially expressed in the same gene symbol or overlapping gene, at least two of the five datasets, were selected (Figure 3). In total, 680 genes were obtained based on criteria of $p < 0.05$ for carrying out the process analysis. Based on this novel approach, 44 genes (Table S1) were identified that overlapped as differentially expressed genes between the predicted target of miR-21 (994 genes) and microarray datasets (680 genes) using a Venn diagram (Table S2, Figure 4).

Identification of predicted target genes for miR-21

In this study, 994 predicted genes as potential target

Table 3. Microarray profiling for differential gene expression in T-cells of MS patients

GSE datasets	p<0.05 significant genes	Up-regulated genes	Down-regulated genes
GSE43591	7502	11	12
GSE13732	14776	25	280
GSE16461	1840	394	1159
GSE78244	3927	154	37
GSE81279	140	6	5

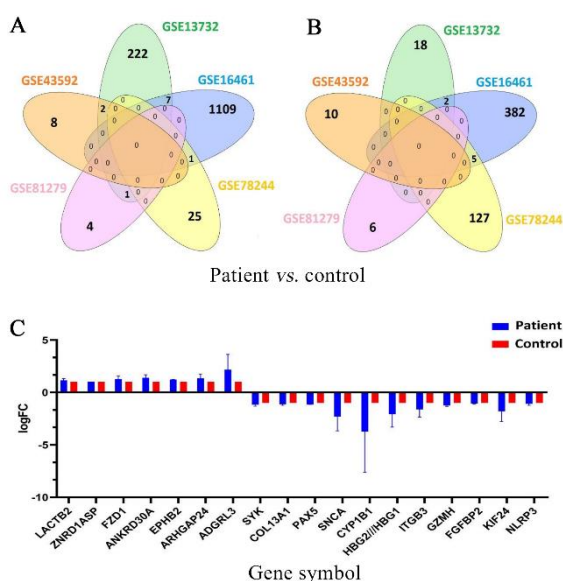


Figure 3. A) Venn diagram represents the number of overlapping differentially down-regulated genes between datasets based on $|\text{Log FC}| < -1$ and $p < 0.05$. Eleven overlapping genes, at least two datasets, were shown. B) Venn diagram represents the number of overlapping differentially up-regulated genes between datasets based on $|\text{Log FC}| > 1$ and $p < 0.05$. Seven overlapping genes, at least two datasets, were shown. C) differentially up- and down-regulated genes between datasets in MS patients versus healthy controls.

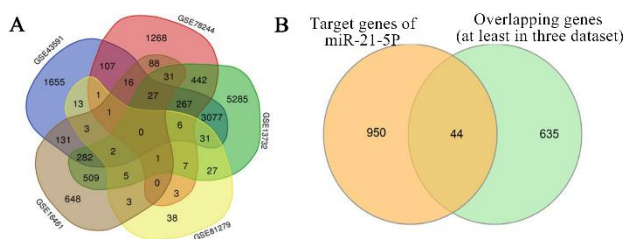


Figure 4. A) 680 overlapping genes, at least three of the five GEO datasets, by Venn diagram with $p < 0.05$. B) The common DEGs (44 genes) as overlapping genes of the predicted target genes of miR-21, at least three of five datasets, using process analysis demonstrated by Venn diagram. miR: microRNA, DEGs: differentially expressed genes, MS: multiple sclerosis.

genes of miR-21 were obtained by using mirDIP. All genes shown in table S1 were predicted by mirDIP as targets of miR-21. Then, the target genes were aligned with the DEGs in MS, and this was used for further analysis.

RNA sequence analysis

Our analysis identified 6332 mRNAs that were significantly differentially expressed between MS and healthy subjects ($p < 0.05$), defined as differentially expressed genes. Then, overlapping genes between these genes and significant genes (44 common genes) and 18 mRNAs ($p < 0.05$, $1 < |\text{LogFC}| < -1$) were shown by microarray analysis (Figure 5).

[KDM7A- ATXN3- SNX13- SMC1A- TMEM106B- WSB1- PIKFYVE- DNAJC16- CAMSAP2- TNFAIP3- ETNK1- IRAK1BP1- RBMS1- U2SURP- ZADH2- NRIP1- RTKN2- BRWD1- AND S100A10]

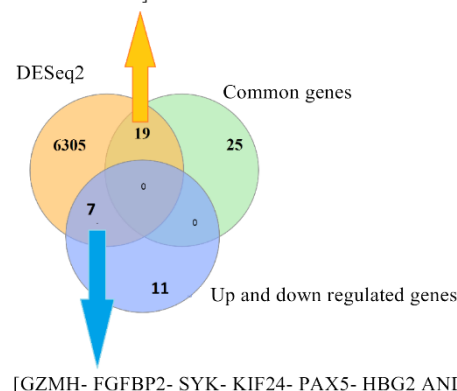


Figure 5. Venn diagram represents the number of overlapping differentially expressed genes between significant genes ($n=6332$) of RNA-seq analysis, 44 common genes and 18 up- and down-regulated genes in multiple sclerosis disease. Validation of microarray result by RNA-seq showed 19 and 7 overlapping genes with common genes and up- and down-regulated genes, respectively.

GO and KEGG pathway enrichment analyses of common genes

GO and KEGG pathway enrichment analyses were performed for further investigation of the functional role of common DEGs and key pathways in MS patients. First of all, all common DEGs which had been submitted to the Enrichr online database were analyzed.

As shown in table 4, signaling pathway analysis was performed using KEGG analysis for all common DEGs (44 genes). The results of KEGG enrichment analysis showed that the common DEGs were mainly enriched in inositol phosphate metabolism, sulfur metabolism, phosphatidylinositol signaling system, HIF-1 signaling pathway, Th17 cell differentiation, and thiamine metabolism. For Cellular Component (CC), results of the top five GO terms (Table 5) reveal that 44 common DEGs were significantly enriched at microtubule minus-end, nuclear periphery, microtubule end, mitotic spindle pole, and membrane raft-mediated pathway (Table S3). For Biological Processes (BP), results of the top five GO enrichment analyses (Table 6) show

Table 4. Significantly enriched KEGG signaling pathways of the differentially expressed genes identified in multiple sclerosis

KEGG pathway	p-value	Genes
Inositol phosphate metabolism	0.011556016	PIKFYVE; IMPAD1
Sulfur metabolism	0.019630393	IMPAD1
Phosphatidylinositol signaling system	0.020048578	PIKFYVE; IMPAD1
HIF-1 signaling pathway	0.020429484	ARNT; IL6R
Th17 cell differentiation	0.023180005	IL6R; TGFB2
Thiamine metabolism	0.032507623	AK2

Table 5. Ten top GO enrichment analyses of 44 common differentially expressed genes (DEGs) with p<0.05

Cellular component pathway ID	p-value	Genes
Microtubule minus-end (GO:0036449)	1/31E-04	<i>NIN; CAMSAP2</i>
Nuclear periphery (GO:0034399)	6/74E-04	<i>ATF7; SMC1A; DCAF7</i>
Microtubule end (GO:1990752)	0/001062505	<i>NIN; CAMSAP2</i>
Mitotic spindle pole (GO:0097431)	0/001374107	<i>NIN; SMC1A</i>
Membrane raft (GO:0045121)	0/002276993	<i>PIKFYVE; S100A10; TGFBR2</i>
Nucleolus (GO:0005730)	0/00344751	<i>ATXN3; NIN; NRIP1; WDFY3; BRWD1; KDM7A</i>
Caveola (GO:0005901)	0/006755543	<i>ATP2B4; TGFBR2</i>
Nuclear matrix (GO:0016363)	0/007474434	<i>SMC1A; DCAF7</i>
Nucleoplasm part (GO:0044451)	0/012080201	<i>PHF20; IMPAD1; ARNT; DCAF7</i>
Gamma-secretase complex (GO:0070765)	0/013129125	<i>TMED10</i>

Table 6. Ten top biological process enrichment analyses of 44 common differentially expressed genes (DEGs) with p<0.05

Biological process pathway ID	p-value	Genes
Protein K63-linked deubiquitination (GO:0070536)	3/13E-05	<i>ATXN3; TNFAIP3; BRCC3</i>
Negative regulation of protein depolymerization (GO:1901880)	8/76E-04	<i>LIMA1; CAMSAP2</i>
Protein K48-linked deubiquitination (GO:0071108)	0/001162071	<i>ATXN3; TNFAIP3</i>
Cellular response to interleukin-6 (GO:0071354)	0/001162071	<i>ST3GAL6; IL6R</i>
Regulation of interleukin-6 production (GO:0032675)	0/003851281	<i>TNFAIP3; IL6R</i>
Regulation of smooth muscle cell proliferation (GO:0048660)	0/004219716	<i>TNFAIP3; IL6R</i>
Negative regulation of supramolecular fiber organization (GO:1902904)	0/006294875	<i>LIMA1; CAMSAP2</i>
Hemopoiesis (GO:0030097)	0/01215973	<i>RTKN2; TGFBR2</i>
Monoubiquitinated protein deubiquitination (GO:0035520)	0/013129125	<i>ATXN3</i>
Regulation of epithelial to mesenchymal transition Involved in endocardial cushion formation (GO:1905005)	0/013129125	<i>TGFBR2</i>

that they were significantly enriched and contained protein K63-linked deubiquitination, negative regulation of protein depolymerization, protein K48-linked deubiquitination, cellular response to interleukin-6, and regulation of interleukin-6 production (Table S4). In addition, according to the results of the top five GO analyses shown in table 7, 44 common DEGs were significantly enriched in Molecular Function (MF), including Lys63-specific deubiquitinase activity, ubiquitin-like protein-specific protease activity, thiol-dependent ubiquitin-specific protease activity, thiol-dependent ubiquitin hydrolase activity, and polyubiquitin modification-dependent protein binding (Table S5).

Construction of protein-protein interaction network

To assess the protein-protein interaction network, all DEGs were submitted to STRING. As shown in figure 6, PPI network analysis introduced 44 nodes and 6 edges for the common DEGs based on the PPI network modules and PPI enrichment with p-value of 0.638.

Recognition of drugs related to common DEGs

Next, an analysis of all the common DEGs using DGIdb v3.0 was carried out to detect affected genes associated with drugs in MS. An in-depth dissection of the effects of drugs on genes in MS was developed. These results demonstrated that seven genes in MS were targeted by drugs. According to table 8, multiple

Table 7. Ten top molecular functions enrichment analyses of 44 common differentially expressed genes (DEGs) with p<0.05

Molecular function pathway ID	p-value	Genes
Lys63-specific deubiquitinase activity (GO:0061578)	1/62E-06	<i>ATXN3; TNFAIP3; BRCC3</i>
Ubiquitin-like protein-specific protease activity (GO:0019783)	5/77E-04	<i>ATXN3; TNFAIP3; BRCC3</i>
Thiol-dependent ubiquitin-specific protease activity (GO:0004843)	6/24E-04	<i>ATXN3; TNFAIP3; BRCC3</i>
Thiol-dependent ubiquitinyl hydrolase activity (GO:0036459)	0/001088282	<i>ATXN3; TNFAIP3; BRCC3</i>
Polyubiquitin modification-dependent protein binding (GO:0031593)	0/004033525	<i>TNFAIP3; BRCC3</i>
Protein phosphatase 2B binding (GO:0030346)	0/013129125	<i>ATP2B4</i>
Transforming growth factor beta-activated receptor activity (GO:0005024)	0/013129125	<i>TGFBR2</i>
1-phosphatidylinositol-4-phosphate 5-kinase activity (GO:0016308)	0/013129125	<i>PIKFYVE</i>
Interleukin-6 receptor binding (GO:0005138)	0/015300881	<i>IL6R</i>
Adenylate kinase activity (GO:0004017)	0/015300881	<i>AK2</i>

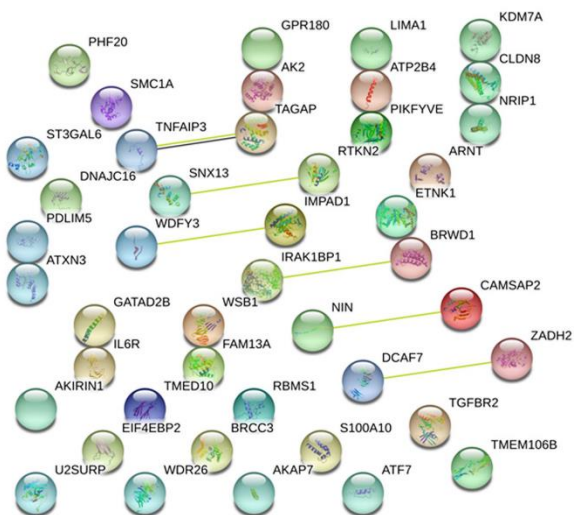


Figure 6. Protein-protein interaction of 44 common differentially expressed genes (DEGs) identified in multiple sclerosis by STRING.

drugs have regulatory and inhibitory roles in MS patients' genes.

Construction of regulatory miRNA-mRNA-drug network

This approach was eventually used to develop a miRNA-mRNA-drug interaction network and identify key genes co-regulated by miR-21-5p and drugs. To illustrate the complex correlation between drugs and gene targets of miR-21, a layered network using Cytoscape v3.6.1 was created that can provide more detailed information regarding these relationships. By integrated analyses, it was shown that 7 genes (*NRIP1*, *ARNT*, *KDM7A*, *S100A10*, *AK2*, *TGFBR2*, and *IL-6R*)

were regulated by obtained drugs and miR-21; in fact, miR-21 and drugs can synergistically regulate pathways in MS disease by regulating these genes (Figure 7).

Discussion

The involvement, functions, and complexity of miRNAs in autoimmune diseases are still unclear, especially in MS, due to the inadequate number of microarray expression profiles in MS studies¹⁹. Overexpression of miR-21 in patients with MS may be a signature in regulating genes and enhanced expression of pro-inflammatory factors such as IFN γ and TNF- α after TCR stimulation. Up-regulation of miR-21 has been found in autoimmune diseases like IBD (Inflammatory Bowel Disease), SLE (Systemic Lupus Erythematosus), and psoriasis. Our findings suggest that miR-21 could be a target in clinical treatment for the inflamma-

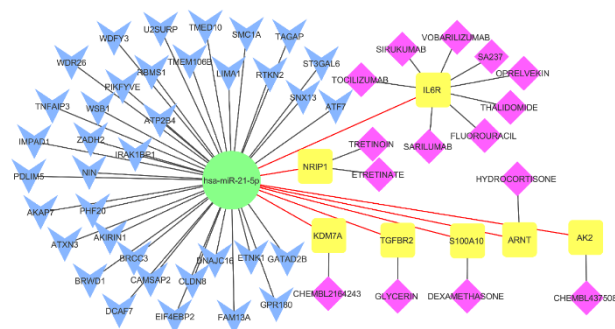


Figure 7. miRNA-mRNA-drug interaction network constructed by Cytoscape; miR-21 regulates common DEGs and is related with genes affected by MS-associated drugs. Blue: common DEGs, Yellow: common DEGs affected by drugs, Pink: MS-associated drugs.

Table 8. Results of the analysis of common DEGs in MS and targeted drugs using DGIdb v3.0

Drug	Interaction type	Gene	Sources	PMIDs	Score
ChEMBL 2164243	Inhibitor	<i>KDM7A</i>	Guide to Pharmacology	None found	1
Glycerin	N/A	<i>TGFBR2</i>	DrugBank	17139284, 17016423	3
Dexamethasone	N/A	<i>S100A10</i>	NCI	10358078	2
Tretinoin	N/A	<i>NRIP1</i>	NCI	15632153, 14581481	3
Etretinate	N/A	<i>NRIP1</i>	NCI	15180561	2
			- My Cancer Genome TGD Clinical Trial - Guide to Pharmacology		
Tocilizumab	Antibody, inhibitor	<i>IL6R</i>	- ChEMBL interactions TEND - DrugBank - TTD	16899109	8
Sarilumab	Antagonist	<i>IL6R</i>	- ChEMBL interactions - TTD	None found	2
Thalidomide	N/A	<i>IL6R</i>	NCI	12515619	2
Fluorouracil	N/A	<i>IL6R</i>	NCI	8888499	2
Oprelvekin	Agonist	<i>IL6R</i>	Guide to Pharmacology	None found	1
Vobarilizumab	Antibody	<i>IL6R</i>	Guide to Pharmacology	None found	1
SA237	Antagonist	<i>IL6R</i>	ChEMBL interactions	None found	1
Sirukumab	N/A	<i>IL6R</i>	TDG Clinical Trial	None found	1
Hydrocortisone	N/A	<i>ARNT</i>	NCI	10048155	2
ChEMBL 437508	N/A	<i>AK2</i>	DrugBank	10592235, 17139284, 17016423	4

tory component of MS²⁴. Also, previous experimental studies have documented that T-cells transfected with miR-21 secreted IFN- γ and TNF- α by affecting promoter regions and have binding sites for several transcriptional factors such as AP-1, STAT-3, MyD88, and NF- κ B²⁹. MiR-21 directly inhibits the expression of PDCD4 that acts as a biomarker in pathogenic T-cell apoptosis and cell proliferation in human SLE. Overexpression of miR-21 can lead to up-regulation of multiple genes which cause inflammation via activation of pathways such as NF- κ B and MAPK⁴¹. miR-21 indirectly regulates Foxp3 expression⁴². Induced miR-21, upon TCR activation, regulates several signaling pathways including ERK, AP-1 and AKT through negative feedback. Activation of these signaling pathways results in increased effector cells and decreases memory T-cell differentiation⁴³. Since predicting promoter region of pri-miR-21 is complex²⁹ and the exact roles of miR-21 are undetermined in MS disease, targeting miR-21 seems to be useful in developing a treatment based on the new approach. In the present study, publicly available microarray databases were used to analyze significantly differentially expressed genes in MS patients and to identify molecular interactions between miR-21-mRNA and drugs for demonstrating biochemical mechanisms related to MS. Therefore, a miRNA and a gene-drug network was created. Our network is different from previous studies in the literature because it is based on specific microarray datasets of T-cells in MS and pathway genes related to drugs. Also, our study identified 44 significantly up- and down-regulated common genes that may reflect the pathology and progression of MS. In this study, 44 new DEGs were found in T-cell MS datasets with overlap between at least three out of five microarray datasets. In the present study, to identify 994 putative target genes of miR-21, miRDIP was used which contained 28 different resources of functional annotation datasets. In addition, to obtain a final list of significant DEGs in T-cells from patients with and without MS, an analysis of five different datasets was performed, which identified 679 MS-associated genes. Integrated analysis between predicted target genes of miR-21 and DEGs of datasets revealed 44 common DEGs as overlapping genes that were associated with the development and progression of MS disease. Our findings revealed 7 up-regulated and 15 down-regulated genes at the intersection of the 44 common DEGs with five datasets that might be targets of miR-21 for the therapeutic approach. Therefore, the detection of putative target genes of miR-21 might identify how this miRNA controls different cell signaling pathways and molecular mechanisms in MS disease. The results of GO annotation revealed that some genes, such as *ATXN3*, *IL6R*, *AK2*, *ARNT*, and *TGFBR2* are mutually and significantly effective between pathways related to MS disease. Also, the results of KEGG pathway enrichment analysis showed that the *IL6R*, *AK2*, *ARNT*, and *TGFBR2* were the most significant

genes in the HIF-1 signaling pathway, Th17 cell differentiation, and thiamine metabolism pathways. Also, previous *in vitro* and *ex vivo* experimental studies have revealed that human Th17 cells were associated with disease activity and downstream pathways in the pathogenesis of autoimmunity⁴⁴ and they play distinctive effector roles in MS patients⁴⁵. In addition, new drugs that targeted TH17 pathway such as Secukinumab (Cosentyx), human IgG1 κ monoclonal antibody against IL-17A, can help in monitoring the disease activity and their potential role in inhibiting Th17 cell differentiation as therapeutic targets in the treatment of autoimmunity disorders⁴⁴ is confirmed based on findings in Experimental Autoimmune Encephalomyelitis (EAE) (MS disease model), and discovery of the biology and function of Th17 in encephalitogenicity⁴⁶. To discover the functions and roles of 44 common DEGs in MS disease, their correlation with MS-related drugs was assessed and regulatory and inhibitory effects of drugs on genes of MS patients were found. These results, based on the scoring criteria, can confirm the findings of GO and KEGG analysis that *IL6R*, *AK2*, *TGFBR2*, and *ARNT* genes are significantly effective in MS disease. These results indicate the potential therapeutic targets of DEGs in autoimmune MS disease. Through integrated analysis of both hybrid miRNA-mRNA drug network with the Cytoscape, this study identified a noticeable relation between miR-21 and genes, indicating that miR-21 could play pivotal roles in regulating pathways and phenotypes of MS. Interestingly, the regulation of *TGFBR2* by miR-21 has been demonstrated by Luo *et al* similar to our analysis²⁴. Moreover, Meira *et al* have reported the significant down-regulation of *TGFBR2* expression in RRMS patients compared to healthy controls⁴⁷. In our analysis, *ARNT* genes were mainly involved in MS disease pathways, whereas Zorlu *et al* showed that this gene is consistently associated with MS in patients at the secondary progressive phase of the disease⁴⁸. *AK2* as a novel apoptotic pathway⁴⁹, the pivotal role of the *AK2* gene in hematopoiesis, and its association with a pathway controlling cell growth and survival were all explained by previous research⁵⁰. Although the exact role of *AK2*, *ARNT*, and *ATXN3* in MS disease has not been studied yet, they be candidate therapies for MS disease. However, the effect of miR-21 on *AK2*, *ATXN3*, and *ARNT* has not been studied in MS disease and requires further investigation. miRNA is an ideal candidate for therapeutic targets due to the role of miRNAs in controlling various gene expression in cancer and several other diseases, in particular autoimmune diseases⁵¹. Nineteen genes among common genes were validated with RNA sequencing in this study. Finally, three overlapping genes (*S100A10*, *NRIP1*, *KDM7A*) were identified between miRNA-gene-drug network and nineteen genes as hub genes that may reflect the pathology of MS. It has been found that *NRIP1* is involved in CNS-mediated neurophysiological processes and ad-

ministration of Toll like-receptor ligands affects inflammatory potential in macrophages through their function as co-activators for NF- κ B⁵². He *et al* have mentioned that methylation is controlled by histone lysine methyltransferases (KMTs) and demethylases (KDMs) that possess strong substrate specificity and they have reported that histone lysine demethylases (KDMs) such as KMD7A play critical roles in the pathogenesis of MS⁵³. It has been identified that S100A10 as the specific marker of A2 astrocytes is essential for cell proliferation, membrane repair, and inhibition of cell apoptosis. Astrocytes play a key role in demyelinating diseases, like multiple sclerosis⁵⁴. Recent data demonstrate that artificial antisense miRNAs, such as Locked Nucleic Acid (LNA), bind to complementary RNA with high affinity and have stability and low toxicity without inducing the immune response⁵⁵; therefore, they could be applied to block their targeted oncomiRs to prevent the development of cancer. Also, antisense miRNAs as a gene silencing factor could significantly affect the prognosis of the disease⁵¹. In particular, LNA against miR-122 represents an effective approach in the treatment of hepatitis C (Phase II trial)⁵⁵.

Conclusion

The computational approach used in this study demonstrated the role of miR-21 as a regulator of the MS-related signaling pathways which can be a potential target for therapeutic modalities. Based on complex miRNA-mRNA interactions, genes targeted by many miRNAs have several sites for the same miRNA. However, the findings of the current study should be confirmed with available techniques such as real-time PCR and western blotting or luciferase assay. Since experimental validation of miRNA targets with laboratory techniques is expensive and cumbersome, the results of current bioinformatic approach would be an effective method for guiding in vivo and in vitro experiments.

An integrated miRNA-mRNA-drug network was developed to analyze predicted MS-associated target genes of miR-21, followed by functional enrichment assessment of the miR-21 targeted DEGs in MS patients. Based on the crucial effect of miR-21 on genes in MS patients, our research suggests applying miR-21 inhibitors such as locked nucleic acid (LNA)-modified oligonucleotides that are known as stable, non-toxic drugs which do not induce an aberrant immune response⁵⁶. Altogether, these findings can provide new insights into pathogenicity mechanisms of MS, therapeutic development, and interventions. Further studies are required to confirm the results of the present study in MS patients.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Conflict of Interest

The authors declare no conflict of interest.

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Supplementary

Table S1. 44 genes were identified that overlapped as differentially expressed genes between the predicted target of miR-21 and microarray datasets

Gene symbol	Gene symbol	Gene symbol	Gene symbol
<i>GATAD2B</i>	<i>S100A10</i>	<i>AKAP7</i>	<i>BRCC3</i>
<i>KDM7A</i>	<i>RTKN2</i>	<i>ATP2B4</i>	<i>LIMA1</i>
<i>TAGAP</i>	<i>AKIRIN1</i>	<i>IRAK1BP1</i>	<i>CLDN8</i>
<i>FAM13A</i>	<i>ETNK1</i>	<i>SMC1A</i>	<i>RBMS1</i>
<i>ST3GAL6</i>	<i>EIF4EBP2</i>	<i>TMEM106B</i>	<i>SNX13</i>
<i>TGFBR2</i>	<i>WSB1</i>	<i>IL6R</i>	<i>ATF7</i>
<i>PIKFYVE</i>	<i>NRIP1</i>	<i>TMED10</i>	<i>GPR180</i>
<i>ZADH2</i>	<i>DNAJC16</i>	<i>PDLIM5</i>	<i>U2SURP</i>
<i>DCAF7</i>	<i>WDR26</i>	<i>BRWD1</i>	<i>WDFY3</i>
<i>TNFAIP3</i>	<i>NIN</i>	<i>ATXN3</i>	<i>CAMSAP2</i>
<i>PHF20</i>	<i>IMPAD1</i>	<i>ARNT</i>	<i>AK2</i>

Table S2. 680 overlapping genes, at least three of the five GEO datasets, with p<0.05

Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol	Gene symbol
CMKLR1	EPB41L3	PLBD1	ARHGAP30	PTBP3	ELOVL5	PIGS	CTNNB1	ITPK1-AS1	KDSR	BDP1	ZDHHC14	RUFY2	IFNGR1	
CYBB	WSB1	CDC73	CENPO	STAB1	NOP9	UBAP2L	IFIH1	CCL28	BCL2L14	IMPAD1	SLC30A5	PDE4A	HLA-DRB1	
CD44	WDR26	ITFG1	MNDA	SSR2	FAM76B	RPL23AP32	TOLLIP	UBE4B	VPS16	OFCC1	TOLLIP	CYP11B	CD5	
TRAF3	FAM13A	GAS7	F11	TPT1-AS1	SEN7	CD81	TICAM1	LYL1	MAST2	GPDL1	LINC00487	BRWD1	IL18	
TLR2	NDE1	LINC01578	CTSS	TRAF3IP3	NDUFC1	INPP5D	INPP5D	WDR5	IKZF5	ADAMTS6	ZC3H10	PANX1	GPR180	GPR180
BCL10	FCGR2A	TIMP2	LPCAT2	DMWD	ITPR1P2	MS4A7	RAF1	RANBP10	GIMAP8	MKKS	EIF3K	EIF3K	ICAM4	
TRIM56	MDC1	SNX24	LMO2	TSPAN17	FAM229A	TBC1D10B	ATG5	MCM3AP	PHF3	SLC25A30	HPS5	TSC22D1-AS1	HTRA1	
PITPNC1	WDFY3	MT1H	CCDC134	MT2A	ZFP41	GATAD2B	ILF3	MFSDB4B	GPATCH2L	MIR6791//GPR108	UBR2	SQSTM1	GDF7	
LINC01210	ZDHHC3	FAM208A	AK2	FLT4	VT1A	XPO5	INIP	UGP2	PLXNC1	CDK13	ARHGAP27	ZNF397	SMAD6	
PPHLN1	PSD4	SRSF5	SKIV2L	RHBDD1	CFAP44	CCL2	CMPK2	APOBEC3G	C10orf76	RPS6KA4	PDE12	ZNF148	NFATC4	
EPHA3	CAMSAP2	TMEM106B	RSBN1L	ARHGFE10L	DNAJC16	ZNF160	PHF20	UCHL5	RPS2P45	LOC100102903	LOC100996385	CNOT4	ABCC3	
BAZZA	FUT11	MS4A14	FAM131A	TNIK	HMOX1	KLC2	QKI	COX4I1	LOC101929219//LOC100505650//C1orf186	DOCK9-AS2	CEP250	IKZF1	SLC6A15	
EXOSC2	FBXL17	RTKN2	AARS2	TPP2	IP6K2	PART1	DCUN1D5	LOC145783//ZNF280D	TRMT44	CD247	FBXO42	BRMS1	TPMT	
ACOX1	ATP2B4	U2SURP	SRPK2	ZSCAN30	EIF5B	SAMD1	NFYC	MAP4K4	GUSBP11	SFT2D3//WDR33	SFT2D3//WDR33	ARNT	LINC00960	
ZNF41	ILDR1	PIKFYVE	STK4	FAM13A-AS1	FLCN	MST1	ZSCAN12	WNKI	C20orf196	CLCN3	CLCC1	BTRC	PEX26	
FGFR2	SLC7A7	HNMT	TGOLN2	CCDC141	DMXL2	POLQ	MCFL2	WAC	ER12	POLR2A	CDC14A	MAU2	BRD2	
LOC283788	CA5B	NECTIN2	ATP6V1G1	CSTF3	CSGALNACT1	CLDN8	ZNF775	LRIG2	PML	SLC35C1	SSTR2	PPP2R1B	TCOF1	
FZD1	FRG1BP	ALDH2	SNX3	COPA	TRNT1	EPHB2	F2RL2	VPS33A	BPTF	RNH1	MIR6824//SLC26A6	FAM208B	PDLIM5	
ETNK1	CSNK2A1	SBN02	RAB5C	USP10	ARSD	ZDHHC8	ELP6	TOP3A	MGA	CCDC71L	LOC101930655//C7orf73//SLC13A4	CEP162	AKAP7	
SLC8A1	SAT1	RAB35	THUMPDI	LY86	TMED10	MICAL3	CANT1	BETIL	FLT3	KIR3DL3	SORT1	ARL10	HIF3A	
PREP	RBMS1	FKRP	IRGQ	CCDC102A	SEC62	ATF7	LOC339803	FRY	MMAA	OVOL1	LOC101929964//LINC01184	NEU3	DST	
SNX25	TSPAN32	APPBP2	BCPIP	RET	TP53	PAK4	TRPM3	BIRC5	HNRNPM	ZNF2	KIR2DL4	TBKBP1	CCNB1	
RAP1A	NID1	CST3	RNASET2	MID1	AZ12	PURG	FAF8	EIF3M	TRIM44	GAK	CELF1	P2RY12	NRXN1	
CD163	HCP5	OPA1	CCND2	LRRC25	INSIG1	TAB1	SNRPB2	POM121L12	SLC45A4	PAX5	DYNC1H1	PYHIN1	P2RY2	
TBL1X	RAB14	VSTM4	ATP5C1	MS4A6A	L3MBTL1	LMX1A	PMS1	TLL11	MTMR2	OSER1-AS1	ADGRG5	WDR78	SYK	
LOC10192704	DYNC1L12	ZNF107	TTPAL	ATXN3	SOS1	CTNNA3	RAB28	DIDO1	IL16	ZNF641	BCAP29	MOGAT2	SOC52	
ABI2	SMCJA	CNO77	BCAT1	CDC42	PACSIN2	CXCR4	GSE1	CARS	TTN	MYO10	IQSEC2	DOT1L	PKHD1	
VPS53	FCGR1B	CTSK	LIG3	PER3	BTG1	GF11	AP2A2	IL27RA	TARP	DLG3	NEK6	SEMA6A	SGCD	
RBM14	RRAS2	DHCR7	TAOK1	CCNT2	CDKN1B	ALAS1	ABTB1	SEN8	NDUFA10	ARIH2	PSMD6-AS2	ATRIP//TRX1	LAMB2	
TUBA1B	ARPC5	TMEM259	GNL3	AIF1	DNAH6	POLR1B	KMT2C	ARFGEF2	LRCH3	AP2A1	BRCC3	ISTNA1	STEAP3	
MROH2B	ANXA6	TEPSIN	MON1B	ARHGGEF40	RHRP1	MAF	TMEM110	ZNF781	TTC9	PLEKHG3	KCND3	PPP1R12B	KIF5A	
RANBP3	BRE-AS1	ZNF862	ANAPC4	TYW3	DNAJC3	CXCL1	ZADH2	EXOSC3	ABHD6	GLB1L2	LRRC42	BACE1	MEGF8	
ZNF322	ST3GAL6	HN1L	TMX4	DDIT4	LIMA1	TNF	EIF4EBP2	LOC389834	PRKCA	PDGFB	SNX13	NUP188	CORO6	
JAK3	SEPT9	HNRNPA2B1	KIAA1033	FAM198B	SIRPB1	XCL1	MIR146A	LIMS1	RDH10	LRPPRC	TMEM185B	NIN	ATP5SL	
NCR1	TCEB3	CLEC7A	AGTPBP1	S1PR1	MPP1	LCK	LOC149684//BPI	SLC25A42	PTCD3	ZBTB7A	UBE2L3	ZKSCAN5	SPIRE1	
C1R	SLC26A11	VPS37A	BRD4	PPME1	GFM1	BCL2L11	PTGDR	KIAA1109	CTAGE5	DCAF7	LCMT2	PRB1	CCDC171	
CCNI	MARCH7	PHACTR2	TNRC18	ACAD10	DIS3L2	TNFSF13B	CDK12	IRAK1BP1	FAM129C	RIMKL3	FAM120AOS	VPS18	ABLIM3	
OSBPL8	MTIX	MAFB	CPVL	S100A10	TMEM41B	CEBPB	MTF1	C5orf63	FOXK2	ACPP	NLRX1	ATP8A1	TGFB2	
NR4A2	NUP50	AKR1C1	TRAM2	UBQLN1	NFAM1	IRAK4	LINC00894	ZNF717	ING5	ZNRF2	B3GALT2	MRPS27	SERPING1	
AP1S2	S100A8	GABBR2	LOC153684	POLR2C	MLX	IL6R	NFKBID	RNF216	POLR2J4	SCARB1	KIDINS220	UBTD2	CD74	
CDK1	TRAPPC10	IGF2R	PPCDC	DERL1	TAX1BP1	TAGAP	OXR1	IKKBK	SPTBN1	HACD3	DDX31	AKAP13	TIRAP	
SETDB1	RAP2B	FCER1G	SULF2	RASGRF2	FAM35A	TNFAIP3	NAGS	ST6GALNAC4	MED27	CIC	DNAJC21	EFCA1B3	HFE	
SAMSN1	HMG2	APEX2	PIEL	CSF1R	C11orf21	CXCL2	KEAP1	CNIH4	NCAM1	HTT	DNM2	SSX2IP		
SEC14L1	PPP1R2	PFKFB4	KMT2B	TMF1	COTL1	IFNAR2	GLYCTK	GLS	GPATCH2	TPGS2	IFT27	PDZD4		
KLF4	LRIF1	SREBF1	PPARD	CRTAM	MARCKS	CD59	PTPN1	DCAF10	NFYA	MRPS5	ZFAND5	TMED1		
AKIRIN1	MED17	PWWF2B	STK10	MTF	VCPKMT	RELA	LINC00528	C9orf40	MAGOHB	STRBP	RNF8	JAK2		
ANKRD26	SPOCK2	CN2M2	ZFAND1	LILRB2	NPEPPS	CD48	NTP160	VRK3	SVIL	PLAGL2	BAALC-AS2	CASP2		
B2M	DIAPH1	ERCC6L2	KDM7A	SLC33A1	CREG1	CD40LG	LOC101928589//TMEM164	TMTC1	TLK1	HNF4A	ZNF91	LTB4R		
SEPHS1	GTF3C4	RAB5B	MT1E	LAX1	SLC9A1	PTK2	C15orf57	ERGIC2	TERF2	CLEC12A	APC	TAL1		

Bioinformatics Analysis of the Potential Regulatory Effects of miR-21 on MS-Associated Drug in Multiple Sclerosis

Table S3. GO enrichment (Cellular component pathway) analyses of 44 common differentially expressed genes (DEGs) with p<0.05

Cellular component pathway ID	p-value	Genes
Microtubule minus-end (GO:0036449)	1.31E-04	<i>NIN; CAMSAP2</i>
Nuclear periphery (GO:0034399)	6.74E-04	<i>ATF7; SMC1A; DCAF7</i>
Microtubule end (GO:1990752)	0.001062505	<i>NIN; CAMSAP2</i>
Mitotic spindle pole (GO:0097431)	0.001374107	<i>NIN; SMC1A</i>
Membrane raft (GO:0045121)	0.002276993	<i>PIKFYVE; S100A10; TGFBR2</i>
Nucleolus (GO:0005730)	0.00344751	<i>ATXN3; NIN; NRIP1; WDFY3; BRWD1; KDM7A</i>
Caveola (GO:0005901)	0.006755543	<i>ATP2B4; TGFBR2</i>
Nuclear matrix (GO:0016363)	0.007474434	<i>SMC1A; DCAF7</i>
Nucleoplasm part (GO:0044451)	0.012080201	<i>PHF20; IMPAD1; ARNT; DCAF7</i>
Gamma-secretase complex (GO:0070765)	0.013129125	<i>TMED10</i>
Meiotic cohesin complex (GO:0030893)	0.013129125	<i>SMC1A</i>
Mitotic spindle (GO:0072686)	0.014709297	<i>NIN; SMC1A</i>
COPI-coated vesicle (GO:0030137)	0.017467967	<i>TMED10</i>
Spindle pole (GO:0000922)	0.023180005	<i>NIN; SMC1A</i>
Nuclear inclusion body (GO:0042405)	0.023941303	<i>ATXN3</i>
Trans-Golgi network transport vesicle (GO:0030140)	0.032507623	<i>TMED10</i>
NuRD complex (GO:0016581)	0.034637697	<i>GATAD2B</i>
pericentriolar material (GO:0000242)	0.034637697	<i>NIN</i>
CHD-type complex (GO:0090545)	0.034637697	<i>GATAD2B</i>
Nuclear body (GO:0016604)	0.046308169	<i>IMPAD1; ARNT; WDFY3; DCAF7</i>
Histone acetyltransferase complex (GO:0000123)	0.047322234	<i>PHF20</i>

Table S4. Biological process enrichment analyses of 44 common differentially expressed genes (DEGs) with p<0.05

Biological process pathway ID	p-value	Genes
Protein K63-linked deubiquitination (GO:0070536)	3.13E-05	<i>ATXN3; TNFAIP3; BRCC3</i>
Negative regulation of protein depolymerization (GO:1901880)	8.76E-04	<i>LIM1; CAMSAP2</i>
Protein K48-linked deubiquitination (GO:0071108)	0.001162071	<i>ATXN3; TNFAIP3</i>
Cellular response to interleukin-6 (GO:0071354)	0.001162071	<i>ST3GAL6; IL6R</i>
Regulation of interleukin-6 production (GO:0032675)	0.003851281	<i>TNFAIP3; IL6R</i>
Regulation of smooth muscle cell proliferation (GO:0048660)	0.004219716	<i>TNFAIP3; IL6R</i>
Negative regulation of supramolecular fiber organization (GO:1902904)	0.006294875	<i>LIM1; CAMSAP2</i>
Hemopoiesis (GO:0030097)	0.01215973	<i>RTKN2; TGFBR2</i>
Monoubiquitinated protein deubiquitination (GO:0035520)	0.013129125	<i>ATXN3</i>
Regulation of epithelial to mesenchymal transition involved in endocardial cushion formation (GO:1905005)	0.013129125	<i>TGFBR2</i>
COPI-coated vesicle budding (GO:0035964)	0.013129125	<i>TMED10</i>
Membrane raft assembly (GO:0001765)	0.013129125	<i>S100A10</i>
Positive regulation of hormone metabolic process (GO:0032352)	0.013129125	<i>ARNT</i>
COPI coating of Golgi vesicle (GO:0048205)	0.013129125	<i>TMED10</i>
Regulation of T cell tolerance induction (GO:0002664)	0.013129125	<i>TGFBR2</i>
Negative regulation of bone resorption (GO:0045779)	0.013129125	<i>TNFAIP3</i>
Aggrephagy (GO:0035973)	0.013129125	<i>WDFY3</i>
Response to DNA damage checkpoint signaling (GO:0072423)	0.013129125	<i>SMC1A</i>
Protein deubiquitination involved in ubiquitin-dependent protein catabolic process (GO:0071947)	0.013129125	<i>TNFAIP3</i>
Golgi transport vesicle coating (GO:0048200)	0.013129125	<i>TMED10</i>
Regulation of cardiac muscle hypertrophy in response to stress (GO:1903242)	0.013129125	<i>ATP2B4</i>
Regulation of intracellular signal transduction (GO:1902531)	0.013642088	<i>PHF20; FAM13A; TAGAP; AKAP7</i>
Negative regulation of nitric oxide biosynthetic process (GO:0045019)	0.015300881	<i>ATP2B4</i>
Regulation of hormone biosynthetic process (GO:0046885)	0.015300881	<i>ARNT</i>
Negative regulation of nitric oxide metabolic process (GO:1904406)	0.015300881	<i>ATP2B4</i>
Response to misfolded protein (GO:0051788)	0.015300881	<i>ATXN3</i>
Regulation of DNA endoreduplication (GO:0032875)	0.015300881	<i>SMC1A</i>
Regulation of toll-like receptor 3 signaling pathway (GO:0034139)	0.015300881	<i>TNFAIP3</i>
Positive regulation of CD4-positive, alpha-beta T cell activation (GO:2000516)	0.015300881	<i>TGFBR2</i>

Table S4. contd.

Biological process pathway ID	p-value	Genes
Regulation of transcription from RNA polymerase II promoter in response to oxidative stress (GO:0043619)	0.017467967	<i>ARNT</i>
Microtubule nucleation by microtubule organizing center (GO:0051418)	0.017467967	<i>NIN</i>
Regulation of amyloid precursor protein catabolic process (GO:1902991)	0.017467967	<i>TMED10</i>
Calcium ion import across plasma membrane (GO:0098703)	0.017467967	<i>ATP2B4</i>
Response to epinephrine (GO:0071871)	0.017467967	<i>ATP2B4</i>
Regulation of toll-like receptor 2 signaling pathway (GO:0034135)	0.017467967	<i>TNFAIP3</i>
Histone H3-K36 demethylation (GO:0070544)	0.017467967	<i>KDM7A</i>
Negative regulation of toll-like receptor 4 signaling pathway (GO:0034144)	0.017467967	<i>TNFAIP3</i>
Positive regulation of alpha-beta T cell differentiation (GO:0046638)	0.017467967	<i>TGFBF2</i>
Negative regulation of monooxygenase activity (GO:0032769)	0.017467967	<i>ATP2B4</i>
Cellular response to epinephrine stimulus (GO:0071872)	0.017467967	<i>ATP2B4</i>
Negative regulation of bone remodeling (GO:0046851)	0.017467967	<i>TNFAIP3</i>
Calcium ion import into cytosol (GO:1902656)	0.017467967	<i>ATP2B4</i>
Regulation of ERAD pathway (GO:1904292)	0.017467967	<i>ATXN3</i>
Proteolysis involved in cellular protein catabolic process (GO:0051603)	0.017827712	<i>ATXN3; TNFAIP3</i>
Protein deubiquitination (GO:0016579)	0.018853327	<i>ATXN3; TNFAIP3; BRCC3</i>
B cell homeostasis (GO:0001782)	0.019630393	<i>TNFAIP3</i>
Atrioventricular valve development (GO:0003171)	0.019630393	<i>TGFBF2</i>
Microtubule anchoring at centrosome (GO:0034454)	0.019630393	<i>NIN</i>
Golgi vesicle budding (GO:0048194)	0.019630393	<i>TMED10</i>
Protein modification by small protein removal (GO:0070646)	0.01963191	<i>ATXN3; TNFAIP3; BRCC3</i>
Membrane raft organization (GO:0031579)	0.021788169	<i>S100A10</i>
Protein K11-linked deubiquitination (GO:0035871)	0.021788169	<i>TNFAIP3</i>
Myeloid dendritic cell differentiation (GO:0043011)	0.021788169	<i>TGFBF2</i>
Microtubule anchoring at microtubule organizing center (GO:0072393)	0.023941303	<i>NIN</i>
Embryonic hemopoiesis (GO:0035162)	0.023941303	<i>TGFBF2</i>
Positive regulation of mesenchymal cell proliferation (GO:0002053)	0.023941303	<i>TGFBF2</i>
Negative regulation of cardiac muscle hypertrophy (GO:0010614)	0.023941303	<i>ATP2B4</i>
Regulation of actin filament depolymerization (GO:0030834)	0.023941303	<i>LIMA1</i>
Response to sterol (GO:0036314)	0.023941303	<i>TGFBF2</i>
DNA repair (GO:0006281)	0.025350515	<i>ATXN3; BRCC3; SMC1A</i>
Regulation of mesenchymal cell proliferation (GO:0010464)	0.026089806	<i>TGFBF2</i>
Signal transduction involved in G2 DNA damage checkpoint (GO:0072425)	0.026089806	<i>BRCC3</i>
Histone H3-K9 demethylation (GO:0033169)	0.026089806	<i>KDM7A</i>
Vesicle budding from membrane (GO:0006900)	0.026089806	<i>S100A10</i>
Response to interleukin-6 (GO:0070741)	0.026089806	<i>ST3GAL6</i>
Positive regulation of vascular endothelial growth factor receptor signaling pathway (GO:0030949)	0.026089806	<i>ARNT</i>
Negative regulation of reactive oxygen species biosynthetic process (GO:1903427)	0.028233687	<i>ATP2B4</i>
Pathway-restricted SMAD protein phosphorylation (GO:0060389)	0.028233687	<i>TGFBF2</i>
Signal transduction involved in DNA damage checkpoint (GO:0072422)	0.028233687	<i>BRCC3</i>
Positive regulation of ERAD pathway (GO:1904294)	0.028233687	<i>ATXN3</i>
Regulation of cAMP-dependent protein kinase activity (GO:2000479)	0.028233687	<i>ATP2B4</i>
Positive regulation of alpha-beta T cell proliferation (GO:0046641)	0.028233687	<i>TGFBF2</i>
Branching involved in blood vessel morphogenesis (GO:0001569)	0.030372956	<i>TGFBF2</i>
Interleukin-6-mediated signaling pathway (GO:0070102)	0.030372956	<i>IL6R</i>
Histone H4-K16 acetylation (GO:0043984)	0.030372956	<i>PHF20</i>
Regulation of Golgi organization (GO:1903358)	0.030372956	<i>CAMSAP2</i>
Cardiac left ventricle morphogenesis (GO:0003214)	0.030372956	<i>TGFBF2</i>
Myeloid dendritic cell activation (GO:0001773)	0.030372956	<i>TGFBF2</i>
Regulation of defense response to virus (GO:0050688)	0.030372956	<i>TNFAIP3</i>
Regulation of synapse organization (GO:0050807)	0.030372956	<i>PDLIM5</i>
Regulation of calcineurin-NFAT signaling cascade (GO:0070884)	0.030372956	<i>ATP2B4</i>
Cytoskeleton organization (GO:0007010)	0.031356737	<i>RTKN2; BRWD1</i>
Atrioventricular valve morphogenesis (GO:0003181)	0.032507623	<i>TGFBF2</i>
Response to cholesterol (GO:0070723)	0.032507623	<i>TGFBF2</i>
Selective autophagy (GO:0061912)	0.032507623	<i>WDFY3</i>
Histone H4-K8 acetylation (GO:0043982)	0.032507623	<i>PHF20</i>
Phosphatidylethanolamine biosynthetic process (GO:0006646)	0.032507623	<i>ETNK1</i>
Embryonic cranial skeleton morphogenesis (GO:0048701)	0.032507623	<i>TGFBF2</i>
Negative regulation of interleukin-2 production (GO:0032703)	0.032507623	<i>TNFAIP3</i>
Endocardial cushion morphogenesis (GO:0003203)	0.032507623	<i>TGFBF2</i>
Positive regulation of glycolytic process (GO:0045821)	0.032507623	<i>ARNT</i>
Mitotic sister chromatid cohesion (GO:0007064)	0.032507623	<i>SMC1A</i>
Response to X-ray (GO:0010165)	0.032507623	<i>BRCC3</i>
Negative regulation of DNA-dependent DNA replication (GO:2000104)	0.032507623	<i>SMC1A</i>
Histone H4-K5 acetylation (GO:0043981)	0.032507623	<i>PHF20</i>
Positive regulation of coenzyme metabolic process (GO:0051197)	0.032507623	<i>ARNT</i>

Table S4. contd.

Biological process pathway ID	p-value	Genes
Negative regulation of translational initiation (GO:0045947)	0.034637697	<i>EIF4EBP2</i>
Sialylation (GO:0097503)	0.034637697	<i>ST3GAL6</i>
Regulation of toll-like receptor 4 signaling pathway (GO:0034143)	0.034637697	<i>TNFAIP3</i>
Ruffle organization (GO:0031529)	0.034637697	<i>LIMA1</i>
mRNA transcription from RNA polymerase II promoter (GO:0042789)	0.034637697	<i>ARNT</i>
Regulation of cell cycle phase transition (GO:1901987)	0.034637697	<i>ATP2B4</i>
Positive regulation of response to endoplasmic reticulum stress (GO:1905898)	0.034637697	<i>ATXN3</i>
Positive regulation of focal adhesion assembly (GO:0051894)	0.036763188	<i>S100A10</i>
Septin ring organization (GO:0031106)	0.036763188	<i>RTKN2</i>
Negative regulation of B cell activation (GO:0050869)	0.036763188	<i>TNFAIP3</i>
Regulation of small GTPase mediated signal transduction (GO:0051056)	0.038004345	<i>FAM13A; TAGAP</i>
Negative regulation of microtubule depolymerization (GO:0007026)	0.038884106	<i>CAMSAP2</i>
Membrane lipid metabolic process (GO:0006643)	0.038884106	<i>ST3GAL6</i>
Regulation of microtubule polymerization or depolymerization (GO:0031110)	0.038884106	<i>CAMSAP2</i>
Positive regulation of carbohydrate metabolic process (GO:0045913)	0.038884106	<i>ARNT</i>
Regulation of amyloid-beta formation (GO:1902003)	0.038884106	<i>TMED10</i>
Regulation of chemokine production (GO:0032642)	0.038884106	<i>IL6R</i>
Acute-phase response (GO:0006953)	0.038884106	<i>IL6R</i>
Negative regulation of calcium-mediated signaling (GO:0050849)	0.038884106	<i>ATP2B4</i>
Cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	0.038994651	<i>CLDN8; TGFB2</i>
Regulation of mitotic spindle assembly (GO:1901673)	0.04100046	<i>SMC1A</i>
Cellular response to interleukin-7 (GO:0098761)	0.04100046	<i>BRWD1</i>
Regulation of protein kinase A signaling (GO:0010738)	0.04100046	<i>AKAP7</i>
Purine ribonucleoside bisphosphate metabolic process (GO:0034035)	0.04100046	<i>IMPAD1</i>
Histone deubiquitination (GO:0016578)	0.04100046	<i>BRCC3</i>
Negative regulation of lymphocyte activation (GO:0051250)	0.04100046	<i>TNFAIP3</i>
Positive regulation of adherens junction organization (GO:1903393)	0.04100046	<i>S100A10</i>
Protein heterotetramerization (GO:0051290)	0.04100046	<i>S100A10</i>
Calcium ion transport into cytosol (GO:0060402)	0.04100046	<i>ATP2B4</i>
Interleukin-7-mediated signaling pathway (GO:0038111)	0.04100046	<i>BRWD1</i>
Negative regulation of innate immune response (GO:0045824)	0.043112259	<i>TNFAIP3</i>
Regulation of vacuole organization (GO:0044088)	0.043112259	<i>PIKfyve</i>
Cellular response to estradiol stimulus (GO:0071392)	0.043112259	<i>NRIP1</i>
Phosphatidylethanolamine metabolic process (GO:0046337)	0.043112259	<i>ETNK1</i>
Calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules (GO:0016338)	0.043112259	<i>CLDN8</i>
mRNA transcription (GO:0009299)	0.045219514	<i>ARNT</i>
Dendritic cell differentiation (GO:0097028)	0.045219514	<i>TGFB2</i>
Regulation of interleukin-2 production (GO:0032663)	0.045219514	<i>TNFAIP3</i>
Ventricular septum morphogenesis (GO:0060412)	0.045219514	<i>TGFB2</i>
Cellular response to misfolded protein (GO:0071218)	0.045219514	<i>ATXN3</i>
Histone lysine demethylation (GO:0070076)	0.045219514	<i>KDM7A</i>
Embryonic skeletal system morphogenesis (GO:0048704)	0.045219514	<i>TGFB2</i>
Cellular macromolecule biosynthetic process (GO:0034645)	0.04668864	<i>EIF4EBP2; ARNT; RBMS1</i>
Regulation of microtubule depolymerization (GO:0031114)	0.047322234	<i>CAMSAP2</i>
Regulation of vascular endothelial growth factor receptor signaling pathway (GO:0030947)	0.047322234	<i>ARNT</i>
Regulation of bone resorption (GO:0045124)	0.047322234	<i>TNFAIP3</i>
Positive regulation of erythrocyte differentiation (GO:0045648)	0.047322234	<i>ARNT</i>
Outflow tract septum morphogenesis (GO:0003148)	0.047322234	<i>TGFB2</i>
Negative regulation of intracellular signal transduction (GO:1902532)	0.048879819	<i>ATP2B4; TNFAIP3</i>
Positive regulation of ATP metabolic process (GO:1903580)	0.049420428	<i>ARNT</i>
Cellular response to catecholamine stimulus (GO:0071870)	0.049420428	<i>ATP2B4</i>
Positive regulation of cell junction assembly (GO:1901890)	0.049420428	<i>S100A10</i>
Membrane assembly (GO:0071709)	0.049420428	<i>S100A10</i>
Regulation of dendritic spine morphogenesis (GO:0061001)	0.049420428	<i>PDLIM5</i>
TOR signaling (GO:0031929)	0.049420428	<i>EIF4EBP2</i>

Table S5. Molecular functions enrichment analyses of 44 common differentially expressed genes (DEGs) with p<0.05

Molecular function pathway ID	p-value	Genes
Lys63-specific deubiquitinase activity (GO:0061578)	1.62E-06	<i>ATXN3; TNFAIP3; BRCC3</i>
Ubiquitin-like protein-specific protease activity (GO:0019783)	5.77E-04	<i>ATXN3; TNFAIP3; BRCC3</i>
Thiol-dependent ubiquitin-specific protease activity (GO:0004843)	6.24E-04	<i>ATXN3; TNFAIP3; BRCC3</i>
Thiol-dependent ubiquitinyl hydrolase activity (GO:0036459)	0.001088282	<i>ATXN3; TNFAIP3; BRCC3</i>
Polyubiquitin modification-dependent protein binding (GO:0031593)	0.004033525	<i>TNFAIP3; BRCC3</i>
Protein phosphatase 2B binding (GO:0030346)	0.013129125	<i>ATP2B4</i>
Transforming growth factor beta-activated receptor activity (GO:0005024)	0.013129125	<i>TGFBR2</i>
1-phosphatidylinositol-4-phosphate 5-kinase activity (GO:0016308)	0.013129125	<i>PIKFYVE</i>
Interleukin-6 receptor binding (GO:0005138)	0.015300881	<i>IL6R</i>
Adenylate kinase activity (GO:0004017)	0.015300881	<i>AK2</i>
Beta-galactoside (CMP) alpha-2,3-sialyltransferase activity (GO:0003836)	0.015300881	<i>ST3GAL6</i>
Type I transforming growth factor beta receptor binding (GO:0034713)	0.017467967	<i>TGFBR2</i>
Aryl hydrocarbon receptor binding (GO:0017162)	0.017467967	<i>ARNT</i>
Glucocorticoid receptor binding (GO:0035259)	0.019630393	<i>NR1P1</i>
Histone demethylase activity (H3-K36 specific) (GO:0051864)	0.019630393	<i>KDM7A</i>
Phosphatidylinositol binding (GO:0035091)	0.020429484	<i>SNX13; WDFY3</i>
Lys48-specific deubiquitinase activity (GO:1990380)	0.021788169	<i>ATXN3</i>
Microtubule minus-end binding (GO:0051011)	0.021788169	<i>CAMSAP2</i>
Eukaryotic initiation factor 4E binding (GO:0008190)	0.021788169	<i>EIF4EBP2</i>
Histone acetyltransferase activity (H4-K16 specific) (GO:0046972)	0.021788169	<i>PHF20</i>
Histone acetyltransferase activity (H4-K5 specific) (GO:0043995)	0.021788169	<i>PHF20</i>
Histone acetyltransferase activity (H4-K8 specific) (GO:0043996)	0.021788169	<i>PHF20</i>
Nitric-oxide synthase binding (GO:0050998)	0.023941303	<i>ATP2B4</i>
Histone demethylase activity (H3-K9 specific) (GO:0032454)	0.028233687	<i>KDM7A</i>
Phosphatidylinositol phosphate 5-phosphatase activity (GO:0034595)	0.028233687	<i>PIKFYVE</i>
Transmembrane receptor protein serine/threonine kinase activity (GO:0004675)	0.030372956	<i>TGFBR2</i>
Calcium-transporting ATPase activity (GO:0005388)	0.030372956	<i>ATP2B4</i>
1-phosphatidylinositol binding (GO:0005545)	0.030372956	<i>WDFY3</i>
Phosphatidylinositol-3,5-bisphosphate phosphatase activity (GO:0106018)	0.032507623	<i>PIKFYVE</i>
Phosphatidylinositol phosphate kinase activity (GO:0016307)	0.032507623	<i>PIKFYVE</i>
Nucleotidase activity (GO:0008252)	0.034637697	<i>IMPAD1</i>
H4 histone acetyltransferase activity (GO:0010485)	0.036763188	<i>PHF20</i>
GTP-Rho binding (GO:0017049)	0.036763188	<i>RTKN2</i>
Protein kinase A regulatory subunit binding (GO:0034237)	0.038884106	<i>AKAP7</i>
Transforming growth factor beta binding (GO:0050431)	0.04100046	<i>TGFBR2</i>
Cadherin binding involved in cell-cell adhesion (GO:0098641)	0.04100046	<i>PDLIM5</i>
Mitogen-activated protein kinase binding (GO:0051019)	0.043112259	<i>ATF7</i>
K63-linked polyubiquitin modification-dependent protein binding (GO:0070530)	0.043112259	<i>TNFAIP3</i>
Sialyltransferase activity (GO:0008373)	0.045219514	<i>ST3GAL6</i>
Protein binding involved in cell-cell adhesion (GO:0098632)	0.045219514	<i>PDLIM5</i>
ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism (GO:0015662)	0.047322234	<i>ATP2B4</i>
Actinin binding (GO:0042805)	0.047322234	<i>PDLIM5</i>
Nucleotide kinase activity (GO:0019201)	0.049420428	<i>AK2</i>