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Original Article

# *hsa-miR-520d-3p* and *hsa-miR-449a* are Candidate MicroRNA Regulators in Multiple Sclerosis

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## Abstract

**Background:** An incapacitating chronic inflammatory neurodegenerative illness, known as multiple sclerosis (MS), is characterized by lymphocyte infiltration into the central nervous system. We aimed to identify specific miRNAs whose altered expression correlates with MS diagnosis and therapy selection, which could be biomarkers for these aspects of the disease.

**Methods:** The GSE21079 dataset was obtained for this study using Geoquery version 2.50.5 from the Gene Expression Omnibus database. The miRNAs exhibiting the highest variance were selected, and a miRNA-miRNA interaction network was constructed through a Bayesian network utilizing the bnlearn R package (version 4.7.1). The adjacency matrix generated from the learned network was subsequently analyzed in the Cytoscape environment. For the workbench lab, whole blood samples were collected from the MS Research Center and Al-Zahra Hospital in Isfahan, Iran, between June 2019 and October 2019. RNA extraction was conducted in the laboratory at Isfahan University. Real-time PCR (RT-PCR) was employed to validate the expression changes of the candidate mirRNAs (*hsa-miR-520d-3p*, *hsa-miR-449a*). The results were analyzed using REST 2009 software.

**Results:** The Notch1 signaling pathway was targeted by *hsa-miR-520d-3p* and *hsa-miR-449a* in MS patients, which led to downregulation of critical genes, such as *LIM* and *SH3 protein 1 (LASP1)*, *Tubulin Alpha1c (TUBA1C)*, and *S100 calcium binding protein A6 (S100A6)*. Furthermore, the results from RT-PCR among 50 whole blood samples, comprising 30 cases of MS and 20 control cases, indicated that the expression levels of miRNA in patients with MS exhibited a statistically significant difference compared to those in healthy individuals, with values of 0.324 for *hsa-miR-520d-3p* and 0.075 for hsa-*miR-449a*. These values correspond to a downregulation of 3.1-fold and 13.3-fold, respectively.

**Conclusion:** The findings indicate that MS patients have lower expression levels of *hsa-miR-520d-3p* and *hsa-miR-449a*.

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**Keywords** ● Central nervous system ● Multiple sclerosis ● Real-time PCR ● Notch1

## What's Known

- MicroRNAs (miRNAs) are small non-coding RNAs that have pivotal effects in biological processes. Identifying the specific expression patterns of miRNAs in various diseases, including autoimmune diseases, reveals their regulatory role. This suggests that miRNAs could serve as both biomarkers and potential therapeutic strategies for treating autoimmune diseases.

## What's New

- In the present study, we examined the effect of two hub miRNAs, *miR-449a* and *miR-520d-3p*, identified from the microarray network. Our analysis of the predicted target genes—*TUBA1C, S100A6,* and *LASP1*— revealed that the expression of all three target genes is affected by these miRNAs.

## Introduction

Neurological ailments are becoming more prevalent in society and are the subject of intense research. Multiple sclerosis (MS) remains a public health problem and a therapeutic

challenge. MS is a complex disease where the immune system infiltrates the central nervous system (CNS), attacking and degrading the myelin sheath. This leads to axonal injury and neurological dysfunction within the CNS.[1] The most prevalent phenotype and the predominant form of the disease, accounting for 85% of MS cases, is relapsing-remitting MS (RRMS). Furthermore, 75% of individuals affected by this condition progress to a more advanced stage characterized by neuronal degeneration, known as secondary progressive MS (SPMS).[2] It is now well-recognized that MS is a complicated multifactorial illness influenced by both hereditary and environmental factors that affect the immune reactions responsible for myelin destruction.[3] One such factor is altered microRNA (miRNA) expression, which has been functionally linked to the pathogenesis of MS.[4]

The CNS is the site of expression for over 60% of the human miRNAs discovered to date.[5] Changes in miRNA expression in the nervous system can result in neurological illnesses such as MS because miRNAs play significant roles in neurogenesis and glial cell function by influencing gene expression at the post-transcriptional level. Further, because they are abundantly expressed in immune cells, miRNAs are expected to have a significant impact on MS.[6] Numerous studies have shown the relationship between changes in miRNA expression and the recurrence and recovery of MS. The mode of miRNA regulation varies depending on the type of miRNA and the tissue in which it is expressed. Of note, several miRNAs have been demonstrated to interact with the Notch1 signaling pathway.[3, 7, 8]

Here, we aimed to examine interactions between miRNAs in the context of MS to identify those that are particularly important in the disease.

## Patients and Methods

*Patients' miRNA Sampling and Ethics Statement*

This study investigates the expression levels of two miRNAs, *hsa-miR-520d-3p* and *hsa-miR-449a*, in the blood of MS patients, exhibiting significant differences compared to healthy controls. The analysis encompasses patients at various disease stages, including RRMS and SPMS. Considering various factors such as the psychological challenges faced by patients, budget constraints, time limitations, availability of skilled personnel and necessary facilities, whole blood samples were collected from the MS Research Center and Al-Zahra Hospital in Isfahan, Iran, between June 2019 and October 2019. RNA extraction was subsequently conducted in the laboratory at Isfahan University.

Before sampling, patients' written informed consent was collected for this study along with a data gathering form comprising details about their name, surname, age, blood type, length of sickness, stage of disease, and other features. With the permission ID: IR.UI.REC.1399.076, it is certified that all techniques were carried out in compliance with the pertinent rules and regulations as well as the ethical principles, national norms, and standards.

All experimental procedures were approved by the Ethics Committee of the University of Isfahan, and informed consent was obtained from all participants before the study.

*Sequencing Data*

The GSE21079 dataset was retrieved from the Gene Expression Omnibus (GEO) database. The miRNAs with the largest variance were retrieved and utilized as an input to train the miRNA regulatory Bayesian network (BN) using bnlearn, an R add-on package (version: 4.7.1;) to remove noise from the data and identify differentially expressed miRNAs (DEMs). The highest variance miRNAs were filtered using the following codes: qt=quantile(t(data1); probes=c(0.0002,0.99)); rows1=apply(t(data1), function(x), any(xqt|x>qt)); data2=t(data1)[, rows1].

*Network Construction*

A miRNA-miRNA interaction network was created using a BN implemented in the add-on R package bnlearn. The Cytoscape-based aMatReader plugin (version 3.8.2) (https://apps.cytoscape.org/apps/amatreader) was then used to study the adjacency matrix that resulted from the learned network in the R environment. Different Cytoscape software plugins, including NetworkAnalyzer (https://apps.cytoscape.org/apps/networkanalyzer) and CytoHubba (https://apps.cytoscape.org/apps/cytohubba), were used to identify topological characteristics and conduct analysis. A thorough set of topological parameters was gathered using the NetworkAnalyzer plugin. Network hubs were located using CytoHubba.

*Extracting the mRNAs-miRNAs Network*

According to our assessments of the literature, *LIM, SH3 protein 1 (LASP1), S100 calcium binding protein A6 (S100A6)*, and *TUBA1C* genes have a role in MS.[9] Therefore, the prediction tools miRmap (https://mirmap.ezlab.org/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/), miRDB (http://mirdb.org/), and TargetScan (http://www.targetscan.org/vert_72/) were used to find miRNAs potentially targeting *LASP1*, *S100A6*, and *TUBA1C* genes. We looked at the many

miRNA sets that influence these three genes (figure 1). From the network of a group of miRNAs impacting each of the three discovered genes (*LASP1, S100A6, and TUBA1C*), two miRNA hubs, *miRNA-449a* and *miR-520d-3p*, were found. The existence and function of these hubs were examined. RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/) was used to determine miRNA–mRNA interactions of our postulated key MS genes. RNAhybrid calculates the minimum free energy hybridization of a long and a short RNA. This program has been used to easily, rapidly, and flexibly predict miRNA targets and can be used to predict miRNA/mRNA hybridization based on a single genome and secondary structure analysis using MFE (minimum free energies).

*In silico Functional Analysis of miRNA Target Genes*

The projected target genes of *hsa-mir-449a* and *hsa-mir-5203p* were identified using TargetScan (https://www.targetscan.org/vert_80/). Panther protein database and Ingenuity Pathway Analysis (IPA) software (QIAGEN Inc., Redwood City, CA; https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/) were used to investigate the functions of the top 100 predicted target genes for each miRNA.

*RNA Extraction*

TRIzol solution (Bio BASIC, Canada) was used for total RNA extraction in accordance with the manufacturer's instructions. The amount needed for cDNA synthesis was calculated using a Thermo Scientific Tm Nanodrop One C (One C model, Thermo Fisher Scientific, United States) spectrophotometer by evaluating the purity and concentration of the RNA. In RNA samples, the 260:280 nm absorption ratio varied from 1.7 to 2.0, indicating good quality. RNAs were kept at -70 °C until cDNA synthesis. Genomic DNA was eliminated using Dnase I (Fermentase Cat # ENO 521, Thermo Fisher Scientific, United States) treatment after extraction and before cDNA synthesis.

*Real Time Quantitative PCR (RT-qPCR)*

The cDNAs synthesized in the previous step were subjected to QRT-PCR using the BON-miR High-Specificity miRNA QPCR Core Reagent Kit (Stem Cell Technology Research Center in Iran [info@bonbiotech.ir]) with specific forward primers for each miRNA. The C/D box snoRNAs (SNORD) gene, which has previously been tested as a suitable transcript (https://bonbiotech.ir), was used as an internal control. The reverse universal primer contained in the BON-miR QPCR kit binds to the sequence added to the miRNAs during cDNA synthesis by the BON-miR RT primer. The specificity of the Real-time quantitative PCR (qPCR) reaction is determined by the forward primer (table 1). All steps of preparing the samples for the Real-time reaction (Chrimo4-Bio Rad Supports Opticon Monitor™ version 3.1, Germany) were performed as far away from the light as possible. The reaction steps were carried out in duplicate in the thermocycler (Bio-Rad-USA) using the program: 95 °C, 2 min, 1 cycle; 95 °C, 5 seconds, 40 cycles; 60 °C, 30 seconds.

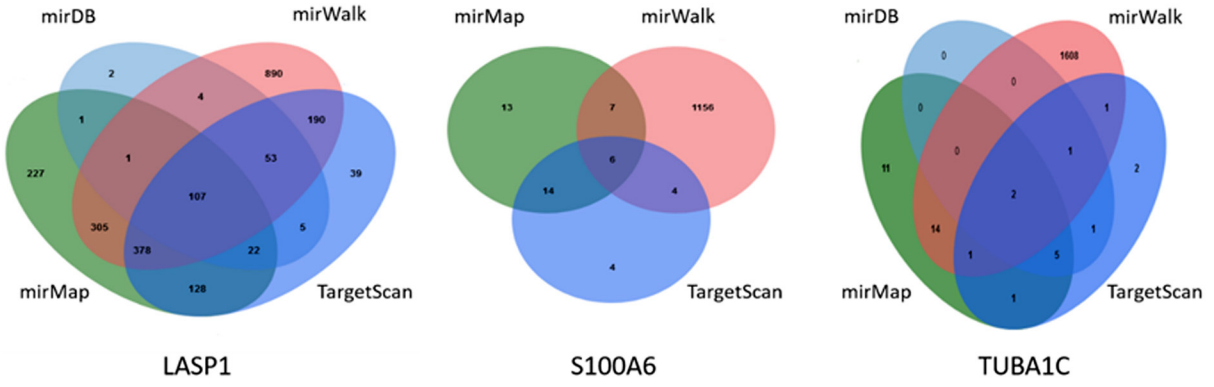


**Figure 1:** Venn diagram showing the overlap of predicted miRNA targets for the three genes *LASP1, S100A6,* and *TUBA1C*. Databases compared were miRmap, miRDB, miRWalk and Target Scan for the two genes of *LASP1, TUBA1C,* and databases miRmap, miRWalk, and Target Scan for the *S100A6* gene. The web tool (https://goodcalculators.com/venn-diagram-maker/) was used to construct the Venn diagram.

**Table 1:** Primers designed for Real-Time Quantitative Reverse Transcription PCR

| No. | miR | Name | Seq(5→3) | TM (°C) |
|---|---|---|---|---|
| 1 | *hsa-miR -520d-3p* | F | GGCTTCTCTTTGGTG | 60 |
| 2 | *hsa-miR-449a* | F | AGATGGCAGTGTATTGTT | 60 |

## Results

*Network Construction*

To identify miRNAs with a role in MS pathogenesis, we made use of a published dataset consisting of miRNA microarray expression profiling in peripheral blood of 59 MS patients categorized into three stages: SPMS with 17 participants, primary progressive MS (PPMS) with 18 participants, and RRMS with 24 participants and 37 healthy controls to learn a BN describing miRNA-miRNA interactions. The learned BN consisted of 732 nodes (genes) and 1,998 edges (interactions) (figures 2A-B). The biological and topological parameters of the network are shown in table 2 and figure 2. In this network, the clustering coefficient was 0.005 and the shortest path amplitude obtained between nodes was between 4 and 7 (figures 2C-D). The clustering coefficient, which is related to the local integrity of the network and calculates the probability of two nodes connecting to a common neighbor, quantifies the richness of connected tri-angles in a learned network and is considered to characterize the internal network structure.

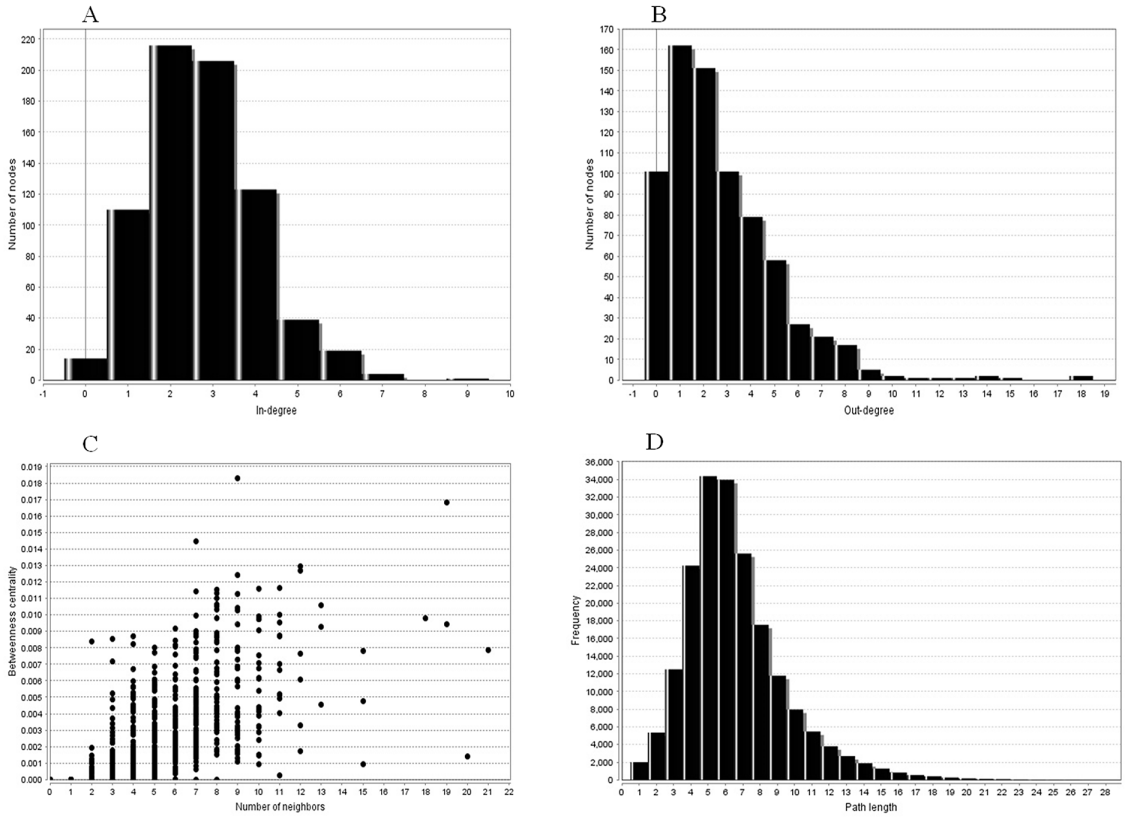


**Figure 2:** The topological measures of the Bayesian network for miRNA expression in MS. A and B: Node degree distribution (number of input and output nodes). C: Distribution of common neighbors. D: Path length.

**Table 2:** Parameters used for microRNA Bayesian network construction with Network Analyzer

| Topological parameters | Values |
|---|---|
| Clustering coefficient | 0.005 |
| Number of nodes | 732 |
| Connected components | 5.00 |
| Network diameter | 27.0 |
| Network radius | 1.00 |
| Shortest paths | 193016 (36%) |
| Characteristics path | 6.524 |
| The average number of neighbors | 5.459 |
| Network density | 0.000 |
| Isolated nodes | 4.00+ |
| Number of self-loops | 0.000 |
| Multi-edge node pairs | 0.000 |
| Analysis time (Sec) | 0.557 |

Cytoscape software-based NetworkAnalyzer plugin

The closer the clustering coefficient is to 1, the greater the probability of cluster formation in the network. The betweenness parameter measures the number of shortest paths passing through a node in the network. However, it is often difficult to determine how the clustering coefficient should be interpreted in a directed network (BN) biologically.

*Identification of miR-449a and miR-520d-3p as Hubs in the Network*

CytoHubba was used to identify several hub miRNAs, of which two (*hsa-miR-520d-3p* and *hsa-miR-449a*) had the greatest connectedness to other hub miRNAs (figure 3). We focused on three target protein-coding genes, *LASP1, TUBA1C,* and *S100A6*, which have been suggested in the literature to have roles in MS pathogenesis. Mature miRNA sequences for *miR-449a* and *miR-520d-3p* were obtained from miRbase (www.mirbase.org), and 3′ UTR sequences for the three target genes from the NCBI gene database. Interactions were examined using the RNAhybrid program, and the most ideal hybridization point between the two sequences was identified based on the projected interaction between the miRNAs (*miR-449a* and *miR-520d-3p*) and several versions of the target gene sequences (*LASP1, TUBA1C,* and *S100A6*). The P value was determined based on the rigid distribution of the normal energy value and the assumption that the target hybridizes and inhibits the pairing of intramolecular bases and branching structures.

The results suggest the *TUBA1C* gene as a significant miRNA target in MS, based on its predicted interaction with the two miRNAs (*miR-449a* and *miR-520d-3p*) and the minimum free energy hybridization.[10] The interaction between *miR-449a* and *miR-520d-3p* with *LASP1* and *S100A6* shows minimal free energy of hybridization.

*Other miRNA Target Genes*

To look for more general functions of the hub miRNAs beyond the three main target genes, we examined the top 100 predicted target genes of *hsa-mir-449a* and *hsa-mir-5203p in silico* to look for related functions. In particular, target genes of *hsa-mir-449a* were enriched for various neurological signaling mechanisms, including the opioid proenkephalin and opioid proopiomelanocortin signaling pathways, dopamine receptor signaling, the 5HT1/2/3/4 signaling pathways, the opioid proenkephalin pathway, the opioid proopiomelanocortin pathway, the pathway for inflammation mediated by chemokine and cytokine signaling, and the transforming growth factor beta (TGFB) signaling pathway. Neurotransmission and neuronal cell differentiation were highlighted as enriched by Ingenuity Pathway Analysis. Together, these enriched functions suggest multiple mechanisms by which *hsa-mir-449a* may contribute to MS pathology.

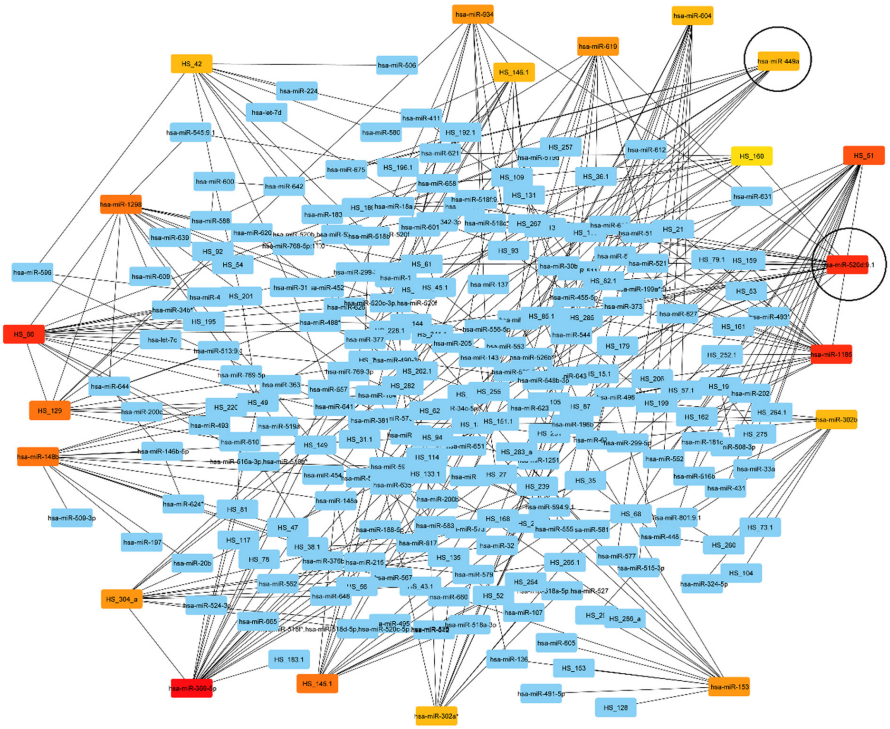

**Figure 3:** CytoHubba results show 20 hub miRNAs as high and low degrees.

**Table 3:** Comparison of *hsa-miR-449a* and *hsa-miR -520d-3p* expression

| miRNA | Relative Expression | Fold change | Std. Error | 95% CI | P(H1) |
|---|---|---|---|---|---|
| *SNORD* | 1.000 | | | | |
| *miR-449-a* | 0.324 | 3.09↓ | 0.084-1.251 | 0.047-7.645 | 0.038 |
| *mir-520d* | 0.075 | 13.3↓ | 0.006-0.627 | 0.003-1.935 | 0.001 |

Relative expression: Expression in MS patients compared to the control group; CI: Confidence Interval; P(H1): Probability of the alternate hypothesis that the difference between sample and control groups is due only to chance; Std. Error: Standard error; Fold change was used to report the data.

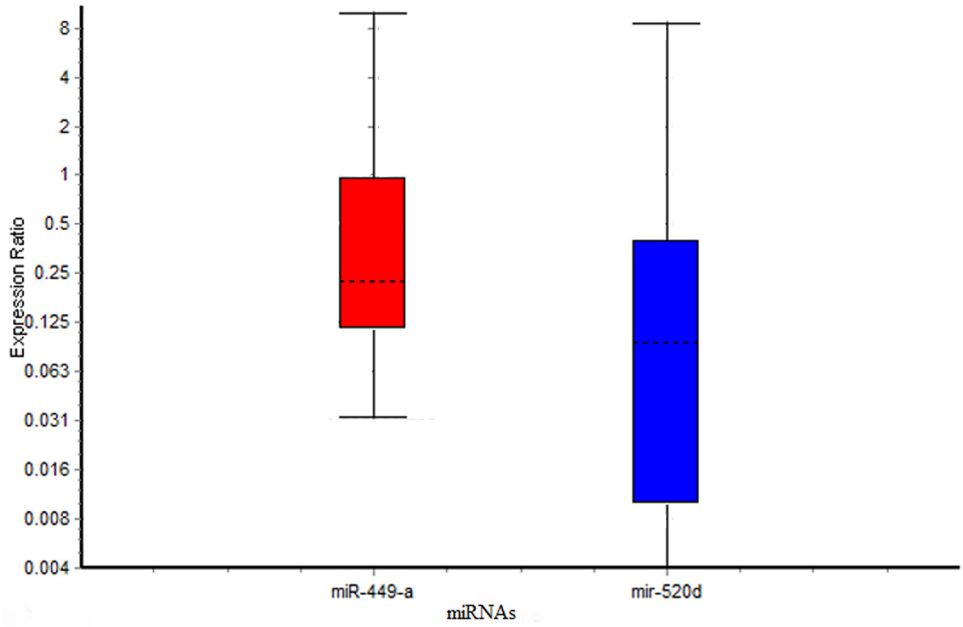


**Figure 4:** Boxes represent the interquartile range or the middle 50% of observations. The dotted line represents the median gene expression. Whiskers represent the minimum and maximum observations.

*miRNA Expression*

A previous work has quantified differences in the expression of *SNORD* reference gene, *hsa*-miR-520d-3p, and *hsa-miR-449a* by RT-qPCR in samples from MS patients compared to healthy controls.[11] Table 3 and figure 4 show the outcomes after analysis with REST2009 software (http://rest.gene-quantification.info/). When MS patients were compared to controls, there was a statistically significant difference between the miRNA expression levels in MS patients and healthy individuals: 0.324 for *hsa-miR-520d-3p* and 0.075 for *hsa-miR-449a*, reflecting 3.1-fold and 13.3-fold downregulation, respectively.

## Discussion

The objective of identifying biomarkers for MS is to facilitate early intervention, tailor patient care, and reduce the costs associated with clinical trials. However, the inherent variability of the disease necessitates the establishment of clearly defined patient populations, dependable assessment platforms, and validation across multiple centers. A combinatorial strategy is essential for developing thorough diagnostic and treatment response profiles.

Numerous investigations have studied miRNA profiling in patients with MS and in control groups, utilizing various sample types such as peripheral blood mononuclear cells (PBMCs), whole blood, and brain lesions. Each of these studies has identified miRNA expression profiles linked to MS. It has been observed that the levels of certain miRNAs diminish due to the influence of oncomirs.[12] In this particular study, the expression levels of *miR-449a* and *miR-520d* were found to be lower than in healthy tissue. The miRNAs *miR-449a* and *miR-520d-3p* regulate gene expression through mechanisms namely post-translational modification, mRNA degradation, or inhibition of translation, and are implicated in various malignancies and neurological disorders. Their loss may impede the development of oligodendrocytes (the CNS's myelin-producing cells),[13] and indeed, a decline in miR-520d-3p expression has been noted in individuals with nervous system problems.[7]

It has recently been reported that miRNAs play a critical role in the Notch signaling pathway, and several miRNAs have been shown

to interact with this pathway, although the exact role of miRNAs in the Notch signaling pathway remains unclear.[7]

*miR-449a* controls the Notch, p53, Wnt, and cell cycle machinery, among other pathways. Of special interest is the finding that *miR-449a* expression is inversely correlated with Notch expression.[8] The Notch signaling pathway prevents oligodendrocyte maturation and myelin production by preventing the differentiation of oligodendrocyte progenitor cells during CNS development. It is believed that MS lesions also activate the Notch signaling system, blocking differentiation of oligodendrocyte progenitor cells to oligodendrocytes and thus impairing myelin production.[1] The surface protein and Notch ligand Jagged1 (JAG1) is one of five cell surface ligands that bind to Notch receptors on oligodendrocytes signaling to inhibit their development.[14]

Research has indicated that cytokine levels in patients with MS fluctuate, resulting in an imbalance between the body's inflammatory and anti-inflammatory processes, which subsequently contributes to heightened inflammation. Consequently, cytokines are recognized as significant factors in MS progression. The *miR-449a* and *miR-520d-3p* promoters are inhibited by secretion of interleukin 13 (IL-13), a cytokine secreted by T helper type 2 (Th2) cells, CD4 cells, natural killer T cell, mast cells, leading to increased signaling through the Notch1 pathway and increased expression of downstream genes, inhibiting oligodendrocyte maturation.[14] Meanwhile, inhibiting the Notch signaling pathway may promote re-myelination.[15]

miRNAs are important to pathogenesis in numerous human diseases, including various types of cancer, infection, chronic inflammations, and autoimmune diseases such as MS.[16] Fingolimod, an immunotherapy medication utilized in treating RRMS, was demonstrated to modify the expression of various coding genes and miRNAs. A prior investigation assessed the expression levels of *miR-449a* in the peripheral blood of MS patients undergoing treatment with Fingolimod, contrasting these levels with those found in healthy individuals. Subjects.[17]

To efficiently identify miRNAs of significant relevance with MS, we developed a miRNA-focused Bayesian network utilizing publicly accessible data that compares miRNA expression profiles between MS patients and healthy individuals. Our analysis concentrated on miRNAs predicted to interact with three genes implicated in MS: *LASP1*, *S100A6*, and *TUBA1C6*. Through this approach, we identified two miRNAs, *miRNA-449a* and *miR-520d-3p*, as central components within the network, and we validated their dysregulation in MS through empirical methods. We anticipate that these two miRNAs play a role in the pathogenesis of MS and may serve as potential diagnostic markers in the future. Further investigation of these miRNAs in both human subjects and animal models will be crucial to clarify the underlying mechanisms, as well as exploring their potential as biomarkers for the diagnosis and treatment of MS.[3, 5, 18]

The collection of blood samples from patients diagnosed with MS presents several challenges, primarily due to the mental and psychological issues faced by these individuals. Consequently, obtaining a substantial number of samples is exceedingly difficult. Furthermore, since MS is a neurological disorder, it is not feasible to acquire brain tissue from living patients. Additionally, studies on MS encounter inherent limitations when attempting to explore subjective experiences through quantitative research methods. To enhance the validity of real-world evidence (RWE) in MS research, an integrative approach that combines various RWE data sources may be beneficial. However, this necessitates the standardization of data collection and processing, along with the establishment of consistent and transparent quality standards.

## Conclusion

Findings show that MS patients have lower expression levels of hsa-*miR-520d-3p* and hsa-*miR-449a*. We predict that these miRNAs may serve as important indicators for disease diagnosis, treatment selection, assessment of treatment efficacy, and prognosis evaluation. However, further research is required to confirm the target genes associated with these miRNAs.

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## Author's Contribution

N.K: Investigation; data curation; drafting. M.M: Formal analysis, investigation, and reviewing the manuscript. M.GhZ: Formal analysis; resources; software, and reviewing the manuscript; BJ. L: Data interpretation and reviewing the manuscript;

All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of Interest:** None declared.

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