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Determination of miRNA Expression Levels Involved in WNT Signaling Pathway in Multiple Sclerosis Patients

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Abstract: Multiple Sclerosis (MS) is an autoimmune central nervous system disease characterized by inflammation, demyelination, and axon damage. Recent studies have shown that the WNT signaling pathway is a negative factor in the process. miRNAs are non-protein-coding RNAs that play a role in processes such as cell development, differentiation, proliferation, and cell death by repressing target genes. As with many pathways, miRNAs are also effective in regulating the WNT signaling pathway. In our study, the expression levels of miRNAs (miR-145, miR-301b, miR214, miR-190a, miR-1304) targeting genes involved in the WNT signaling pathway were examined. Our study was carried out in order to comprehend the relationship between MS and the WNT signaling pathway, to contribute to the clinic and the literature in elucidating the etiology of MS, and determining treatment strategies with the results to be obtained. Blood samples were taken from patients with MS (17) included in our study during both attack and remission periods. Blood samples were taken from the control group (16) participating in the study, and the expression levels of miRNAs included in our study were quantitatively analyzed using the RT-PCR method. When compared with the control group, no statistically significant difference was observed in terms of fold increase values in the miRNA levels (miR-145, miR-301b, miR-214, miR-190a ve miR-1304) of the MS attack period, while statistically significant differences (respectively; p=0.010, p=0.023, p=0.002, p=0.006, p=0.003) were found in terms of fold increase values of all miRNA levels in the remission period. Considering the medications used by the patients and the number of attacks, there was no statistically significant difference in miRNA expression levels. In our study, it was deduced that miRNA expression levels, which are effective in the WNT signaling pathway, may play a role in elucidating the clinical course and genetic mechanism of MS, particularly during the remission period. © 2022 NTMS.
Keywords: miRNA; Multiple Sclerosis; WNT Signaling Pathway.

1. Introduction

Multiple Sclerosis (MS) is defined as an autoimmune disease involving the central nervous system, characterized by demyelination, axonal damage, and inflammation. The etiology of MS disease has not been

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fully elucidated. Myelin sheaths, oligodendrocytes, axons, and nerve cells are damaged. There are many types of signal transduction pathways involved in the development and repair of oligodendrocytes (1). One of the signaling pathways is the evolutionarily conserved WNT signaling pathway. The WNT signaling pathway is significant in the adhesion of cells capable of renewing themselves in adulthood, in the control of transcription of target cell genes, and in maintaining cell polarity and proliferation, cell differentiation, and migration in the embryonic period (2). literature studies demonstrate that the WNT signaling pathway is a negative factor in the myelination process.

miRNAs are non-protein coding RNAs. They are encoded by genes that are transcribed from DNA but not transformed into protein. miRNAs can be found in exonic, intronic regions of protein-coding genes and in intergene regions. miRNAs play a role in processes such as cellular development, differentiation, proliferation, and death by suppressing one or more target genes (3).

The WNT signaling pathway plays a negative role in the myelination process in the CNS. Myelin loss and impaired axonal conduction elicit various neurological deficits such as numbness, weakness, visual defect, and paresis. Damaged myelin can be repaired or remyelinated, consistent with clinical remission. Remyelinated sheaths are susceptible to subsequent demyelination and are characterized by recurrent remyelination and demyelination, clinical relapse, and remission in MS. As a result, it can lead to irreversible disability. To target the autoimmune inflammatory mechanism of MS in the peripheral and CNS, current medications for this disease are immunomodulators. Immunomodulatory therapy is important in alleviating inflammation. They can stop the course of demyelination and prevent clinical exacerbation. However, approaches to support repair for pre-established demyelinated lesions are still deficient. The role of the WNT signaling pathway in the myelination process has been demonstrated. Oligodendrocytes are cells that make remyelination in the CNS and the effects of the WNT signaling pathway on the development of oligodendrocytes have been indicated in studies. Considering its relationship with inflammatory and autoimmunity, studies with the WNT signaling pathway MS will contribute to the literature in order to develop approaches that will lead to timely and effective remyelination (4-6).

As with many cellular signaling pathways, it has essential functions in the regulation of the WNT signaling pathway. miRNAs are very significant in regulating the functioning of all kinds of signaling pathways in our bodies. As a result of the increase or decrease in the expression levels of miRNAs, the functioning of the signaling pathways is affected. Defects in signal pathways play a role in the etiology of many diseases, particularly cancers and neurodegenerative diseases.

Five miRNAs that have a function in the WNT signaling pathway were included in the study. miRNA-145.2 regulating the expression of the FZD7 gene acting on the Frizzled (FZD) receptor miR-301b regulating the expression of the TCF4 gene acting on the TCF/LEF transcription factor miR-214.4 acting on the 3 β catenin protein regulating the CTNNB1 gene MiR-190a, which regulates the expression of the MAPK8 gene acting on MAPK signal, and miRNA-13046, which regulates the expression of the WNT3A gene, acts on the 5 WNT ligand, were included. It was aimed to determine whether there was a genetic relationship between miRNAs involved in the WNT signaling pathway and MS disease.

2. Material and Methods

2.1. Materials

This study was approved by the Atatürk University Faculty of Medicine Clinical Research Ethics Committee (06/15-30.11.2017). Research and Publication Ethics were followed at all stages of the study. The study included 17 MS patients who were admitted to the Atatürk University Health Research and Application Center Neurology Outpatient Clinic, who was in the attack phase and were hospitalized and received attack treatment. A written informed consent form was obtained from the patients. Detailed systemic and neurologic examinations were performed. Clinical, laboratory, and medication information were recorded. Their blood was taken into an EDTA tube. The patients were called for control 3 months after their remission period, and detailed examinations were again made and their blood was taken into an EDTA tube. Demographic data of the patient and control groups in our study is given in Table. 1.

Furthermore, 4 (23.6%) of 17 RRMS patients were newly diagnosed and had not started medication treatment yet, and received solely attack treatment. Five (29.4%) of our patients were using subcutaneous interferon beta-1b (IFN-β-1b), 3 (17.6%) were using subcutaneous glatiramer acetate, and 5 (29.4%) were using oral fingolimod. The average number of attacks of the patients is 5.29. The mean disease duration of our MS patients is 3.35 years.

Table 1: Demographic data of groups.

	MS	Control
Number of patıents/controls	17	16
Age	28.64±7.34	28.81±6.70
Gender F/M n(%)	12(70.6%)/5 (29.4%)	11(68.75%)/5 (31.25%)

2.2. Methods

2.2.3. Quantitative real-time PCR

miRNA was isolated from peripheral blood samples using the miScript RNeasy Mini Kit (Hilden, Germany) according to the manufacturer's instructions and its quality was assessed by spectrophotometric analysis (Maestrogen, MaestroNano Spectrophotometer, USA). cDNA was then synthesized by reverse transcription from 2 µg of total RNA using the QiagenmiScript II Reverse Transcription Kit (Hilden, Germany) with a Labcycler Thermal Cycler (SenSoquest). Diluted cDNA was used as a template for quantitative real-time polymerase chain reaction (RT-PCR) analysis.

The cDNA was used in combination with QiagenmiScript SYBR Green PCR Kit (Qiagen, Germantown, MD, USA) and miScript primer assays. Quantitative rt-PCR was run in a Rotor-Disc 72 with 25-µl reaction volumes for 40 cycles of 95°C for 2 min, 94°C for 15 s, 55°C for 30 s in a QiagenRotorgene Q (Qiagen, Hilden, Germany). The reaction mixture contained 12.5 µL of miScript SYBR Green Master Mix, 1 µL of each primer (forward and reverse primer), 6.5 µL of DNase/RNase-free distilled water, and 5 µL of cDNA template. SNORD61 was chosen as the reference gene for this study. Reference sequence numbers for all primers were obtained from the GenBank.

2.3. Statistical Analysis

Changes in gene expressions of miRNAs were calculated. The 2ΔΔCt analyzes of all miRNAs were analyzed using the GeneGlobe Data Analysis Center online analysis program by entering data into the Excel program of the Ct values given in the Real-Time analysis. SNORD61 98was accepted as reference genes and delta Ct values were calculated first. The p-value was calculated with the Saturn T-test in the same program. A $p< 0.05$ was considered significant. These values were made for all genes included in our study. Descriptive statistics for continuous variables are tabulated as mean and standard deviation.

3. Results

A total of 50 blood samples were taken from the patient and control groups included in our study. cDNA synthesis and enrichment were performed by isolation of miRNA from the collected samples. The expression levels of the miRNAs that we determined (miR-145, miR-301b, miR-214, miR-190a, and miR-1304) were quantitatively identified. According to the statistical analysis; fold differences were observed for miRNAs (miR-145, miR-301b, miR-214, miR-190a, miR-1304) compared between groups compared to the control group.

When miRNA expression levels of the control group were accepted as 1, an increase was observed in 3 miRNA levels (miR-145, miR-214, and miR-1304) of Group 1, while a decrease was observed in 2 miRNA levels (miR-301b and miR-190a). On the other hand, in accordance with the fold increase analysis made by accepting the miRNA expression levels of the control group as 1, all miRNA expression levels of Group 2 were increased. Numerical data of the fold increase is shown in Table 2.

Table 2: Fold increase values between miRNA expression levels of study groups miRNA.

miRNA type	Group 1* fold increase	Group 2* fold increase
hsa-miR-145	1.3431	3.435
hsa-miR-301b	0.7887**	1.4385
hsa-miR-214	1.2509	3.0889
hsa-miR-190a	0.9727**	1.7895
hsa-miR-1304	1.7727	3.7293

*The fold increase values were compared to the control group.
** Fold decrease was observed instead of fold increase.

In the statistical analysis, the significance of the change between the Attack period (Group 1) and Remission (Group 2) MS patients and the control group was evaluated over the 2' Delta CT (2-ΔCT) values normalized with the SNORD61 control.

When compared to the control group, no statistically significant difference (respectively; p=0.833, p=0.704, p=0.738, p=0.759, and p=0.274) was observed in terms of fold increase values in all miRNA levels (miR-145, miR-301b, miR-214, miR-190a ve miR-1304) of Group 1, while a statistically significant difference was found in terms of fold increase values of all miRNA levels of Group 2 (respectively; p=0.010, p=0.023, p=0.002, p=0.006, and p=0.003) (Table.3).

Table 3: P values of the groups and statistical significance.

miRNA type	Group 1 p value	Group 2 p value
hsa-miR-145	0.833	0.010*
hsa-miR-301b	0.704	0.023*
hsa-miR-214	0.738	0.002*
hsa-miR-190a	0.759	0.006*
hsa-miR-1304	0.274	0.003*

*Expresses statistical significance.

The significance of the change between the MS patients in the Attack and Remission period and the control group in the statistical analyzes we made according to the medications used is given in Table 4.

According to the data obtained, of miRNA types analyzed in Group 1, up-regulation was observed in miR-145, miR-214, and miR-1304 expression levels, and down-regulation in miR-301b and miR-190a expression levels. Up-regulation was observed in the expression levels of all miRNA types analyzed in Group 2. The medications used did not have an effect on miRNA gene expressions in our study.

Table 4: P values and statistical significance according to the number of attacks.

ATTACK	miR-145		miR-301b		miR-214		miR-190a		miR-1034	
At S.	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
≥6	0.836	0.016*	0.744	0.019*	0.711	0.004*	0.723	0.007*	0.235	0.002*
2-5	0.872	0.029*	0.706	0.029*	0.821	0.003*	0.753	0.006*	0.291	0.003*
1	0.790	0.013*	0.823	0.019*	0.711	0.001*	0.717	0.005*	0.391	0.002*

G1: Group1, G2: Group2 *Indicates statistical significance.

Table 5: P values and statistical significance according to the drugs used.

	miR-145		miR-301b		miR-214		miR-190a		miR-1034	
DRUG	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
IFN-β-1b	0.887	0.011*	0.77	0.027*	0.749	0.004*	0.769	0.006*	0.238	0.002*
FTY720	0.902	0.017*	0.713	0.021*	0.724	0.005*	0.787	0.006*	0.127	0.003*
GA	0.822	0.022*	0.692	0.032*	0.830	0.002*	0.743	0.007*	0.280	0.003*
OTHER	0.790	0.013*	0.823	0.019*	0.711	0.001*	0.717	0.005*	0.391	0.002*

G1: Group1, G2: Group2 *Indicates statistical significance.

4. Discussion

MS is one of the most common neurological diseases affecting the CNS, with attacks of inflammation in the brain and spinal cord and demyelination of the myelin sheaths surrounding the axons, with multifactorial etiopathogenesis and often affecting young adults. Demyelination often occurs as a result of chronic inflammation in the CNS. In recent years, it has been shown that the WNT signaling pathway has an important role in myelination and remyelination (7).

In this context, we conducted our current study in order to examine the relationship between the WNT signaling pathway, which is involved in the development and repair of oligodendrocytes, and MS disease, and to contribute to the etiology and treatment of MS with possible results. While genetic studies on Multiple Sclerosis have increased significantly in the last 10 years, there are limited studies in the current literature on the role of miRNAs in the development of MS (1, 4, 6-9).

MiR-145 was expressed at a higher rate in the blood samples taken during the attack and remission periods of RRMS patients compared to the control group. While the increase in miR-145 expression level was not significant in Group 1, the expression level was found more than 3 times in Group 2 and it was found to be statistically significant. In the expression study by Sondergaard et al. in the literature, in addition to the fact that miR-145 is expressed 3 times more in MS patients compared to healthy patients, they stated that miR-145 can be used as a possible diagnostic biomarker of miR-145, which can be found in serum and plasma in Peripheral Blood Mononuclear Cells (PBMC). In the study conducted by Keller et al., consisting of 20 MS patients and 19 healthy individuals, 866 different miRNA profiles were examined. They found that 10 miRNA types, including miR-145, were dysregulated in MS patients. As a result of their analysis, they reported that 9 miRNA types, including miR-145, were overexpressed in MS patients, and down-regulation was detected merely in miR-20b. 112 It has been shown that the up-regulation of miR-145, which is known to have a role in the WNT signaling pathway, its up-regulation 122 detected in MS patients has been shown to regulate the differentiation of oligodendrocytes by targeting the FZD7 receptor, which interacts with the WNT signaling pathway (10-11**).** The expression levels of miR-301b and miR-190a were different from each other in Group 1, Group 2, and Control groups. In both miRNA types, downregulation was detected in the attack period of MS patients compared to the control group. Up-expression level was identified in Group 2 compared to both Group 1 and the control group. As a conclusion of analyzes performed on blood samples taken during the remission period, it was ascertained that miR-301b and 38 miR-190a were expressed 1.4385 and 1.7895 times, respectively. In the literature, a hierarchical cluster graph is presented with down-regulation of miR-190 between 0.6 and 2-fold in the control group and up-regulation between 0.2 and 2-fold in the MS patient group (12-13). In another study, it was stated that the expression of miR301b, which is in the miR-130 family in MS patients, induces the release of TNF-alpha and IFN-gamma, thereby negatively affecting the brain functions.

MiR-214, another miRNA type evaluated in our study, was expressed at a higher rate in samples taken from patients in both attack and remission periods compared to the control group. Two different studies also demonstrated that ovarian expression of miR-214 in oligodendrocytes has a significant role in remyelination and axon regeneration (14).

No study has been found in the existing literature on the relationship between miR-1304 included in the study and MS disease. Considering the miR-1304 expression

value detected in both Group 1 and Group 2 in our study, it is understood that there is over-expression.
When the data obtained from other miRNAs in our study is analyzed, we encounter the miRNA with the highest fold increase in both Group 1 and Group 2 compared to the control group (15).
The number of publications in the literature on the relationship between immunomodulatory therapies used in MS and miRNAs is quite limited. There are some studies on the relationship between IFN-Beta-1b (IFN-β-1b), Fingolimod (FTY720), and Glatiramer Acetate (GA) treatments and miRNA expression levels in MS patients (16-17).

5. Conclusions

It is seen that there has been a significant increase in the number of studies on MS-miRNAs in recent years. As a result of these studies, it is predicted that the disease arises as a result of the interaction between environmental stimuli, susceptibility to disease, and determining genes. In our study, the effects of treatments applied to patients on miRNA profiles were analyzed, however statistically significant results were not found. Our study will shed light on future studies on the investigation of miR-145, miR-301b, miR-214, miR-190a, and miR-1304 miRNAs, which are involved in the WNT signaling pathway, which may have significant roles in the development of MS, as well as on the analysis of the course and mechanism of the disease.

Limitations of the Study

There are two major limitations in this study that can be addressed in future research. First, the sample size is larger. Second, the miRNAs of other target genes identified in the wnt signaling pathway inclusıon in the study

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None

Conflict of Interests

The authors declare that there is no potential conflict of interest for the research, authorship, and/or publication of this article. All authors read and approved the final manuscript.

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Author Contributions

Design of the study: EB, Sample collection: NB, Performed the experiments: EY, Data Collection and/or Processing: EB, NB, EY, Writing Original Manuscript: EB, NB, EY. EB contributed to revising the work and final approval of the final version of the manuscript.

Ethical Approval

This study was approved by the Atatürk University Faculty of Medicine Clinical Research Ethics Committee (06/15 30.11.2017).

Data sharing statement

The data that support the findings of this study are available on request from the corresponding author.

Consent to participate

Consent was obtained from the patient and control groups participating in the study.

Informed Consent

The patient and control group who agreed to participate in the study signed the informed consent form.

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