Original Article

Initial Evaluation of lncRNA A2M-AS1 Gene Expression in Multiple Sclerosis Patients

Shaghayegh Mohammadi[1], Tahereh Sadeghiyan[1], Mohammad Rezaei[2], Mansoureh Azadeh[3]

[1]Department of Genetics, Faculty of Biology Sciences and Technology, Shahid Ashrafi Esfahani, Isfahan, Iran, [2]Department of Biology and Biotechnology, University of Pavia, Pavia, Italy, [3]Zist Fanavari Novin Biotechnology Institute, Isfahan, Iran

Abstract

Background: Multiple sclerosis (MS) is one of the three leading neurodegenerative diseases worldwide. Gene expression profile studies play an important role in recognizing and preventing disease. Considering the inherent ability of biomarkers to diagnose and prognose the occurrence of a disease, with the aim of gene therapy and changing gene expression, it can be helped to treat it. In this study, by examining the gene interaction and expression of non-coding genes in patients with MS, using bioinformatics analyses, laboratory research and potential non-coding diagnostic biomarkers of MS were selected for further investigations.

Materials and Methods: First, by using micro-array data analysis of the GEO database, the expression status of the long non-coding ribonucleic acid (RNA) (lncRNA) A2M-AS1 gene was investigated in patients with MS. lncRNA–mRNA interaction analysis was performed in the lncRRisearch database. After sample collection, the total RNA extracted using the RNA extraction kit from 20 patient samples and 20 healthy samples was synthesized into cDNA with the synthesis kit. The quantitative reverse transcriptase polymerase chain reaction experiment was performed for the final validation of expression change.

Results: Based on bioinformatic and laboratory analysis, the expression of the A2M-AS1 gene in MS samples showed a significant decrease in expression compared to healthy samples. Also, based on the receiver operating characteristic analysis, lncRNA A2M-AS1 can be introduced as an acceptable diagnostic biomarker to distinguish MS samples from healthy samples.

Conclusion: lncRNA A2M-AS1, by reducing its expression as an acceptable diagnostic biomarker, can increase the risk of developing MS.

Keywords: Biomarkers, gene expression profiling, long non-coding RNA, multiple sclerosis

Address for correspondence: Dr. Mansoureh Azadeh, Provincial General Office of Technical and Vocational Training Organization, Hezar Jerib Street, Isfahan, Iran.
E-mail: mazadeh@phd.iaurasht.ac.ir
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Introduction

Neurodegenerative diseases (NDDs) are a wide range of diseases that initially involve the neurons of the brain, and over time, the process of progressive damage to the nerves leads to the gradual loss of the structure or function of the neurons and finally the apoptosis of the central nervous cells. Neurodegenerative diseases can affect a person's movements, speech, memory, intelligence, and other things. These diseases are very complex; the exact cause of many of them is still unclear, and because there is no known way to reverse the progressive degeneration of neurons, they are considered incurable.[1]

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system that is caused over time by auto-immune invasion and progressive destruction of the myelin sheath of nerve axons. While the cause is unclear, genetics and environmental factors are proposed as causes.[2,3] Specific genes that have been linked with MS include differences in the HLA system on chromosome 6 that serve as MHCs. The most

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consistent finding is the association between MS and alleles of the MHC defined as *DR15* and *DQ6*. Other loci have shown a protective effect, such as *HLA-C554* and *HLA-DRB1 * 11*.[4] To help early diagnosis and prognosis of MS, vitamin D dose,[5] pathobiological tests of serum or blood–brain barrier fluid and enzyme-linked immunosorbent assay (ELISA),[6] interpretation of demographic data,[7] analysis of neuroimaging data with machine learning,[8] magnetic resonance imaging (MRI),[9] and molecular and genetic biomarkers[6] play an emergent role.

Biomarkers are biological molecules present in all body tissues that indicate a normal or abnormal process, a condition, or a disease. In various cancers and neurodegenerative diseases, at the genetic level, which is called a genetic marker, it is a deoxyribonucleic acid (DNA) sequence that causes or aggravates the disease or is related to the diagnosis and declaration of susceptibility to a specific disease.[6] Previous studies revealed that different long non-coding ribonucleic acids (lncRNAs) might have important regulatory effects on the development of immune-related diseases, such as cancer.[10-15] Hao and colleagues[16] in 2022 found different lncRNAs with an important role in MS pathways, including *C17orf82, BOLA3-AS1, RBM26-AS1, RBMS3-AS3, CRNDE, FAM13A-AS1,* and *VENTXP1*.[16] The given biomarker in this study was lncRNA *A2M-AS1*. LncRNA is transcripts longer than 200 nucleotides that are not translated into protein.[17] LncRNA A2M-AS1 is the number 1 anti-sense RNA of the protein-coding A2M gene (alpha-2-macroglobulin), which has the highest expression level in normal lung and liver cells and is an almost innovative lncRNA in the field of research, which is believed to play a role in cancer pathways. Based on NCBI, the product of this gene is a protease inhibitor and cytokine transporter that inhibits a wide range of proteases. Mutations cause alpha-2-macroglobulin deficiency.

The prevalence of MS has increased in recent decades, and this may be due to the increasing sensitivity of radiographic methods and diagnostic criteria and to longer survival. Since the cause of the development of MS is still unclear, as a result, definitive treatment and reliable prognostic methods have not been provided to improve this disease. Considering the wide range of studies that have shown the innate potential of biomarkers in the prognosis of many diseases, it is possible to take advantage of the potential of the biomarker investigated in this study in the process of better diagnosis in patients. However, the existing gap is that the number of diagnostic biomarkers in MS is very low and they do not have a very accurate and decisive diagnostic application.[18,19] Since no previous study related to the role of A2M-AS1 with MS was found and according to the analyses performed in high-throughput data, it was shown that this molecule had significant expression changes, so this gene was assumed to be a suitable candidate in our study which makes our study novel in this field.

In this research, based on investigating the mechanism of effect and interaction of lncRNA A2M-AS1 with other genes in patients with MS, it is hoped to find a new biomarker in the diagnosis and prognosis of this disease.

Materials and Methods

Gene expression analyses

Gene expression analyses were performed based on micro-array data obtained from the GEO database and RNA sequencing data in the study of GSE43591 to measure and find genes with expression changes (DEGs) in 20 samples of MS patients in comparison to 20 control samples. The platform used in this experiment, which is also known as GPL520, is as follows: [HG-U133_Plus_2] Affymetrix Human Genome Array U133 Plus 2.0. The platform used in this study includes a large and acceptable number of mRNAs and lncRNAs involved in MS with specific expression and covers a wide range of samples, and this platform's data have higher quality than other platforms. In contrast, other platforms do not cover all or most of the LNCs involved in MS or do not include many diverse samples, so this platform was used during the study. The ethics committee approval has been obtained. This study used the blood samples of patients with MS in a survey with IR.UI.REC.1402.016 ethical approval from the ethics committee/IRB of the University of Isfahan on 2/30/1402.

DEG analysis was performed by using affy and limma packages, which were downloaded from the Bioconductor database. At first, to check the quality of the existing samples, quality control was done by performing various tests. Then, by using the linear model available in the Lima package, the mean expression of all control and MS samples was simultaneously measured and compared to each other, and after calculating the difference of each group means, the log fold change was obtained, and the Lima package performed a *t*-test on the obtained data to calculate the *P* value and adjusted *P* value.

Micro-array analysis graphs were drawn using the ggplot2 package, where genes with logFC > 1 and logFC < -1 were identified as significant DEGs with a significance level of adjusted *P* value less than 0.05. All stages of DEG analysis were performed on micro-array data, and corresponding graphs were drawn using the Studio R (Version 4.1.2) programming language.

Sampling

This case-control study was conducted without the direct presence of patients and on 40 blood samples obtained from people with MS and healthy people, according to the modified criteria of McDonald's. Healthy samples of volunteers without any inflammatory or auto-immune disease according to gender, age, and ethnicity were selected as the control group. This case-control study was conducted without the direct presence of patients and on 40 blood samples obtained from people with MS and healthy people, according to the modified criteria of McDonald's. Healthy samples of volunteers without any inflammatory or auto-immune disease according to gender, age, and ethnicity were selected as the control group.

RNA extraction

The total RNA of blood samples was extracted by using the RNX-plus extraction kit. After the removal of genomic DNA

in the extracted RNA, the RNA concentration was estimated by using a Nanodrop Spectrophotometer (ND-1000, ThermoFisher, MA, USA). Purified RNA was stored at -80˚C for further steps.

cDNA synthesis and real-time PCR

Two pairs of forward and reverse primers were designed with oligo7 software for both A2M-AS1 and GAPDH genes, where GAPDH was considered as a control primer. Primer information are provided in Tables 1 and 2. Due to the instability of RNA, to measure the expression level of the target genes, we must use the reverse transcription technique [real-time polymerase chain reaction (PCR)]. The reverse transcription reaction is a reaction in which the RNA-dependent DNA polymerase enzyme converts RNA into cDNA. Treated RNA with DNase turned into cDNA using a Universal cDNA Synthesis Kit (Roje, Iran/Tehran) based on the manufacturer's protocol. The synthesized cDNAs were stored at -20˚C until the PCR. All reactions were carried out in double. Quantitative reverse transcriptase PCR (qRT-PCR) data were assessed according to the -ΔΔCT method. The corresponding lncRNA Ct values were normalized against GAPDH as a reference gene.

Statistical analysis

Statistical analysis of real-time PCR data and graphing were performed using Graph Pad Prism software (Version 9.0.0). Kolmogorov–Smirnov test has been used to measure the normality of the expression data distribution. Paired and unpaired t-tests were used to compare and analyze the difference in the expression of lncRNA A2M-AS1 between control and MS samples. Receiver operating characteristic (ROC) analysis and drawing of the ROC diagram were done by using GraphPad prism software. This test is used to measure the biomarker potential of candidate genes based on two factors including area under curve (AUC) and *P* value. The Mann–Whitney U test was carried out to analyze the quantitative expression level of lncRNAs between patients and healthy groups. Spearman's rank correlation test was performed to evaluate the possible correlation between the relative expression levels of lncRNAs and the clinical data. A *P* value less than 0.5 is considered statistically significant.

Results

Micro-array data analysis

The analysis of micro-array data to investigate the expression changes of long non-protein-coding RNA in patients with MS was performed by R Studio software. Based on the analysis and investigations among the 35,214 genes examined in GSE43591, 62 genes had a significant decrease in expression and 32 genes had a significant increase in expression. Figure 1 shows the top 20 genes with significant increases and decreases in expression in GSE43591. Among the studied genes, A2M-AS1 non-coding long RNA (logFC: -1.796157, adj.P. Val: 0.01884) had significant expression reduction in MS samples compared to healthy ones [Figure 2]. This gene showed a biomarker potential to distinguish disease samples from healthy types, and a real-time PCR test to confirm their expression changes determines the exact amount of biomarker potential of this gene.

According to the strong interactions and expression changes in MS, lncRNA A2M-AS1 is considered the target of this study. In the next steps, the interactions of mRNAs with lncRNA A2M-AS1 were investigated in the LncRRIsearch database, and the highest interacted mRNAs were found [Table 3]. Then the interaction of miRNAs with targeted lncRNA was estimated in the LncBase database and the strongest miRNAs were extracted [Table 4]. For further investigations about proteins and signaling pathways that are related to this lncRNA, protein network interaction with RPGR mRNA as the strongest mRNA interacted with A2M-AS1 lncRNA was found in the STRING database as shown in Figure 3. The respective signaling pathways of these proteins were extracted from the Enrichr database [Table 5].

Gene ontology

Gene ontology of protein network interaction with mRNA RPGR, in this study, was done in three fields including biological process, cellular component, and molecular functions. Biological process includes Cilium Assembly, Plasma Membrane Bounded Cell Projection Assembly, Organelle Assembly, Renal System Development, Positive Regulation of Cell Junction Assembly, Positive Regulation of Intracellular Protein Transport, and Positive Regulation of Nucleocytoplasmic Transport. Cellular components are Cilium, Sperm Flagellum, Cell-Cell Junction, Specific Granule Lumen, and Microtubule Cytoskeleton. Molecular functions are pre-miRNA Binding, Glycerophospholipid Flippase Activity, GTPase Inhibitor Activity, Kinesin Binding, and GDP Binding.

Real-time PCR data analysis

Both designed primer sequence details are shown in

Table 1: Information of forward (F) and reverse (R) primers designed for lncRNA A2M-AS1

Tm (C°)	length (nt)	The amount of water needed to make a 100-micromole solution	Sequence (5'-3')	Primer
58.39	22	222.73	CCTTGTGGTCTTCAAGCATTTC	A2M-AS1 (F)
60.25	22	197.62	TCTGCTCATGGAGACCCAAATC	A2M-AS1 (R)

Table 2: GAPDH control forward (F) and reverse (R) primer information

Tm (C°)	Length (nt)	The amount of water needed to make a 100-micromole solution	Sequence (5'-3')	Primer
58.83	19	224.92	ACAGGGTGGTGGACCTCAT	GAPDH (F)
59.35	20	213.52	AGGGGTCTACATGGCAACTG	GAPDH (R)

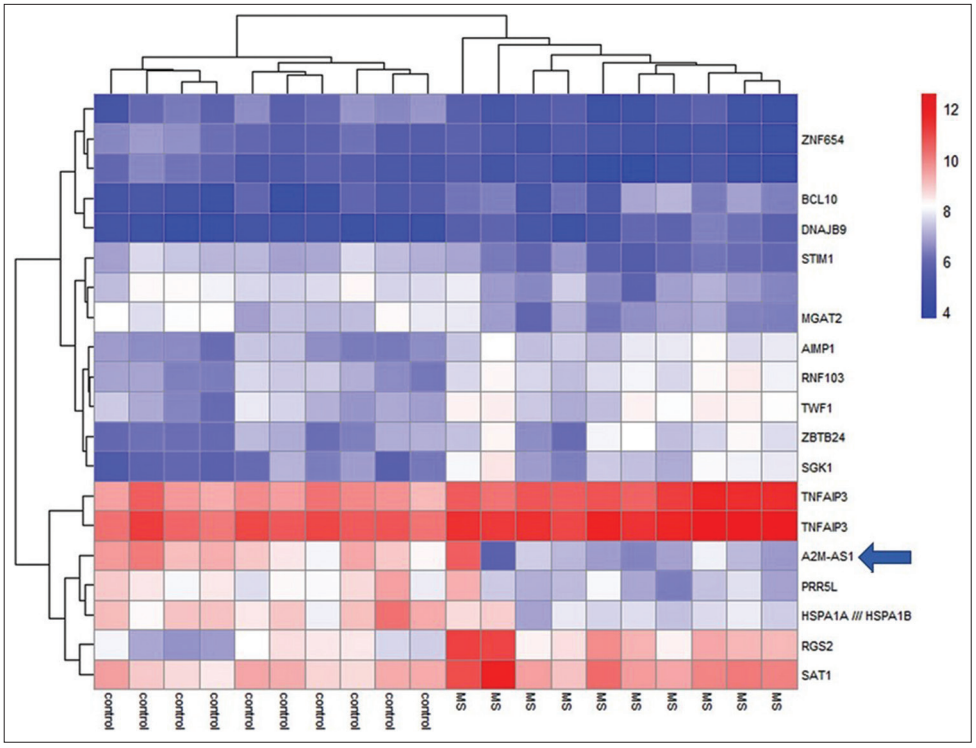

Figure 1: Heatmap diagram of the top 20 genes with increased and decreased expression in MS samples compared to healthy samples. The A2M-AS1 gene is indicated by arrows in the heat map diagram. Based on the image, this gene has a lower expression level in MS samples than in healthy samples

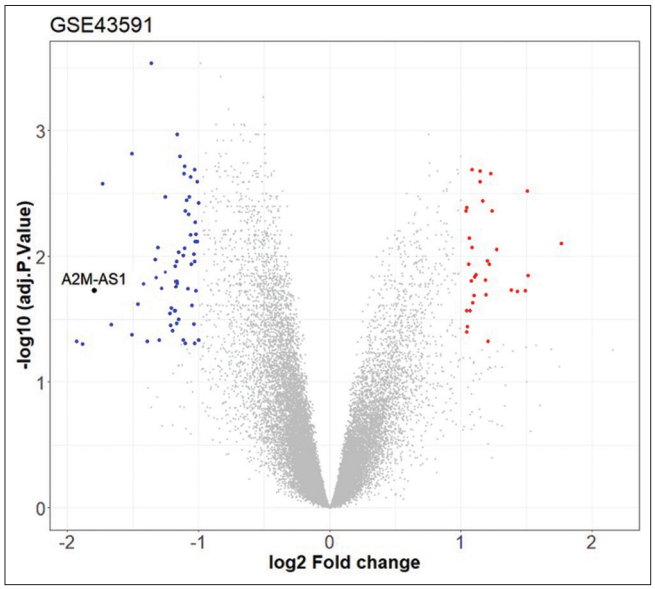

Figure 2: Volcano plot shows genes with increased expression, decreased expression, or no significant expression change. Genes with logFC greater than 1 are identified as genes with increased expression and genes with logFC less than -1 as genes with decreased expression. The significance level of this analysis is an adjusted *P* value smaller than 0.05. Based on the analysis, A2M-AS1 had a significant decrease in expression

Tables 1 and 2. The statistical analysis of the data obtained from the results obtained in the Q-PCR test and the design of the relevant graphs were done using Graph Pad Prism software (Version 9.0.0) and the examination of gene expression changes (DEGs) including -ΔΔCT.

Analysis of expression changes

Analysis of expression changes was done by real-time PCR test to evaluate the changes predicted by micro-array analysis. Based on the analysis, lncRNA A2M-AS1 had a significant expression reduction in MS samples compared to healthy samples (logFC: -2.550, *P* value: 0.0074) [Figure 4]. This result shows the accuracy of the results obtained through micro-array analysis and strengthens the hypothesis of diagnostic biomarkers of the A2M-AS1 gene. ROC analysis was performed to investigate the biomarker potential of this gene. Based on ROC biomarker analysis, A2M-AS1 Lnc RNA (AUC: 0.7075, *P* value: 0.0248) is considered an acceptable potential diagnostic biomarker to distinguish MS patients from healthy samples [Figure 5]. According to Mann–Whitney analysis to investigate the relationship between the expression level of lncRNA A2M-AS1 and patients' ages [Figure 6] and the patients' pathophysiological characteristics [Figure 7], no significant relation was found.

Discussion

Based on the tests and analyses performed in this study, it was shown that in the micro-array data analysis, the A2M-AS1 gene has a significant decrease in expression in MS samples compared to the control. The real-time PCR test also confirmed the results of the bioinformatics analysis and showed that the expression of this gene in MS samples has a significant decrease in expression. These results make the hypothesis of a diagnostic biomarker of this gene stronger. Based on ROC statistical analysis performed on real-time PCR data,

Table 3: Ten mRNAs that interact with lncRNA A2M-AS1. Chosen considering that this lncRNA has strong interactions and has expression changes in MS

Sum of Energy	Min of Energy	Transcript ID	Transcript Name	Type	Genomic Distance
-2,329.61	-41.51	ENST00000378505	RPGR-009	mRNA	10,000,000,000
-1,553.99	-29.40	ENST00000622895	GOLGA6L22-002	mRNA	10,000,000,000
-1,383.99	-37.61	ENST00000222990	SNX8-001	mRNA	10,000,000,000
-1,348.21	-29.42	ENST00000619213	GOLGA6L6-001	mRNA	10,000,000,000
-1,258.59	-34.63	ENST00000334976	EMC10-001	mRNA	10,000,000,000
-1,039.04	-28.50	ENST00000295851	ABI2-001	mRNA	10,000,000,000
-933.24	-28.10	ENST00000614055	GOLGA6L1-001	mRNA	10,000,000,000
-927.05	-44.10	ENST00000397609	FAM98B-001	mRNA	10,000,000,000
-788.60	-28.46	ENST00000567107	GOLGA6L2-002	mRNA	10,000,000,000
-544.62	-35.25	ENST00000380243	CCDC85C-001	mRNA	10,000,000,000

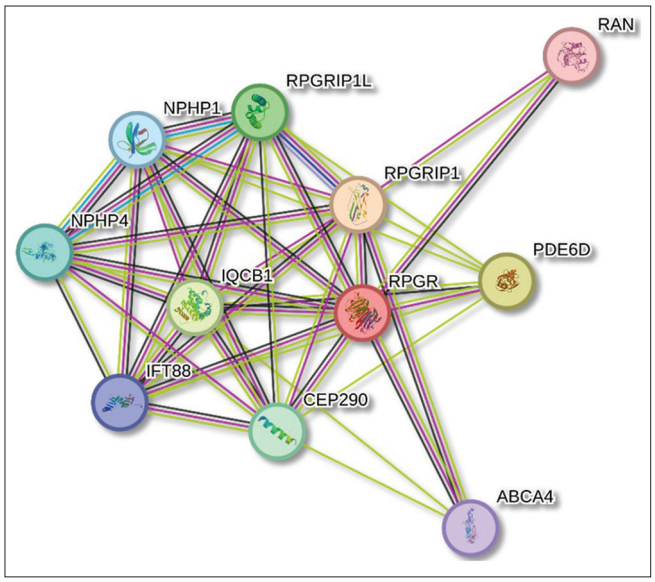

***Figure* 3:** Protein network interactions with RPGR mRNA as the strongest mRNA interacted with A2M-AS1 lncRNA

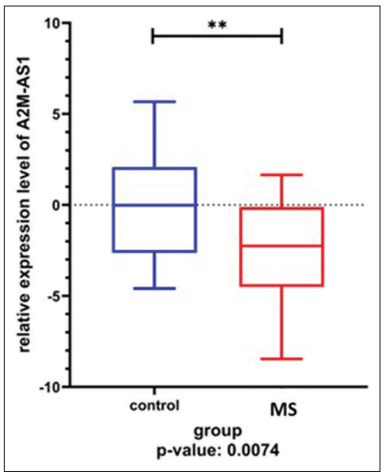

***Figure* 4:** Examining the expression changes lncRNA A2M-AS1 RNA in MS samples compared to healthy ones. Based on this analysis, this considered gene showed a significant decrease in expression in MS samples compared to controls

lncRNA A2M-AS1 can be identified as an excellent diagnostic biomarker.

MS is one of the most significant neurodegenerative diseases that endanger the lives of many people worldwide every year. The rate of MS disease was reported by WHO in 2022 to be about 2.8 million people worldwide and 100 cases out of every 100,000 people in Iran. The high and progressive rate of this disease, as one of the three common diseases and neurodegenerative indicators in the world, due to the lack of definitive treatment, requires extensive clinical research and more bioinformatic and pathological studies. Investigating the status of gene expression profiles plays a very important role in the recognition, management, control, and prevention of various diseases, including neurodegenerative diseases. This study investigates the interaction and expression of non-coding genes in MS patients by using bioinformatics analyses and laboratory research to find the gene expression pattern and the interaction of potential biomarkers of this disease and find appropriate treatment goals that are of great importance. In this study, we investigated the mechanism of effect and interaction of lncRNA A2M-AS1 in patients with MS.

According to the research of Yao and colleagues[20] in 2021, it was shown that lncRNA A2M-AS1 targets genes related to ferroptosis and plays a role in gastric cancer by increasing the expression of ferroptosis and activating CD4+ T-cells.[20] Recent studies and clinical research on this molecule have shown that changes in the expression of the lncRNA A2M-AS1 gene in the occurrence of lung adenocarcinoma,[21] the decrease in its expression in pancreatic cancer,[22] the incidence of multiple myeloma, and the development of cervical cancer are involved. Also, in Wang's studies in 2020, this gene was mentioned as a diagnostic biomarker of acute coronary syndrome, which plays an important role in this disease with its reduced expression[23] the same as this study's result that confirmed the potential biomarker role and its decline expression in MS. In Hong's studies in 2022, it was shown that this lncRNA is a positive regulatory factor with an increased expression pattern to promote breast cancer metastasis.[24] Through the interaction with PCBP3,[25] increasing the expression in the diagnosis of

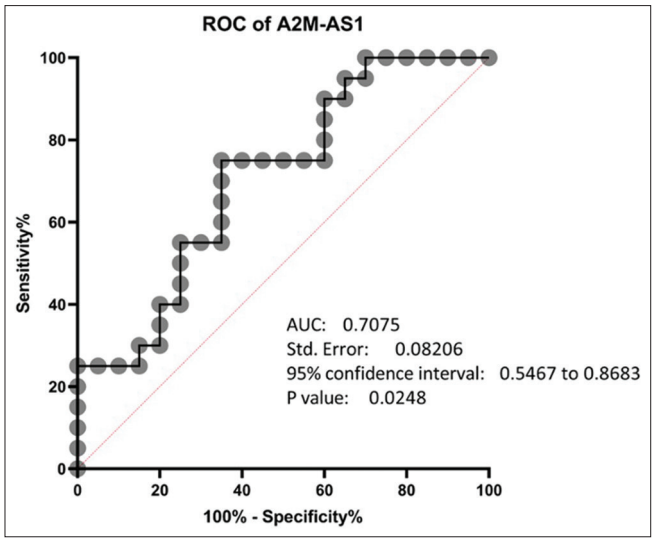

Figure 5: ROC analysis to investigate the biomarker potential of the examined gene in the study. Based on this analysis, A2M-AS1 is an acceptable diagnostic biomarker for distinguishing MS patient samples from healthy samples

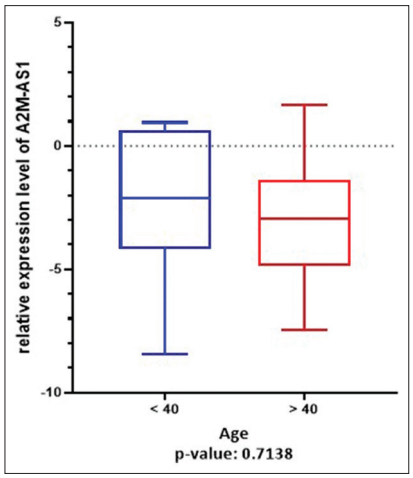

Figure 6: Negative results from Mann–Whitney analysis to investigate the relationship between the expression of lncRNA A2M-AS1 and pathophysiological information of patients including their age. In this analysis, the data between the two types of RRMS and SPMS of MS patients with pathophysiological indicators of the age of 40 and under were analyzed and no correlation was found

Table 4: 10 miRNAs that interact with lncRNA A2M-AS1

miRNA	MirBase ID
hsa-let-7a-5p	MIMAT0000062
hsa-miR-34a-5p	MIMAT0000255
hsa-miR-377-3p	MIMAT0000730
hsa-miR-7-5p	MIMAT0000252
hsa-miR-98-5p	MIMAT0000096
hsa-miR-106a-5p	MIMAT0000103
hsa-miR-181a-5p	MIMAT0000256
hsa-miR-19a-3p	MIMAT0000073
hsa-miR-210-3p	MIMAT0000267
hsa-miR-26a-5p	MIMAT0000082

progression and prognosis of breast cancer[26] and decreasing the expression level of A2M-AS1 in the lung tissue of patients with COVID-19 are severe compared to control subjects.[27] In the studies of Guo in 2022, it was shown that the over-expression of A2M-AS1 through the regulation of miR-587/bmp3 inhibits cell growth and invasion in lung adenocarcinoma.[26] Fang *et al*.[26] in 2020, regarding the role of this RNA in breast cancer, reported that positive regulation and increased expression of A2M-AS1 promotes invasion and migration in this type of cancer and is a poor prognostic biomarker in breast cancer.[26] In this field, Liu's research in 2020 also acknowledged that over-expression of A2M-AS1 lncRNA increases tumor growth by reducing the expression of miR-146b in breast cancer.[21] The comprehensive analysis of lncRNA-A2MAS1 regulatory networks in Alzheimer's disease in 2022 by Li showed that this lncRNA directly affects the main mechanisms of Alzheimer's occurrence, which include autophagy and apoptosis.[28] By following similar signaling pathways, the possible and similar effect of this lncRNA in MS may be addressed too.[16]

Also, in the studies of Panahi, it was shown that the allele of the TPH gene rs17110747 is significantly enriched in Iranian patients with MS, which is an important reason for the high incidence rate of this disease in Iran.[29] According to the different patterns of the gene expression profile of lncRNA A2M-AS1 in different diseases that were mentioned compared to its expression profile in MS, it can be concluded that the expression changes in different diseases have different roles in signaling pathways that can suppress or aggravate a disease. Since there are no clear signaling pathways and much information about the exact effect mechanism of genes in neurodegenerative diseases, especially MS, due to the involvement and participation of an A2M-AS1 lncRNA in the mTOR-PI3K AKT signaling pathway in other neurodegenerative diseases such as Alzheimer which was mentioned earlier, it can be inferred that it has a synergistic or regulatory effect in MS as well. It is suggested to investigate the mechanism of effect of this lncRNA on other more genes in MS disease; future research should be carried out.

In this research, for the first time, an attempt was made to take an effective step in the early diagnosis and prognosis of this disease to investigate the changes in the expression of genes in MS by relying on the mentioned biomarker.

Conclusion

Based on the tests and analyses performed in this study, it was shown that A2M-AS1 lncRNA in the micro-array data in MS samples has a significant decrease in expression compared to control samples. Real-time PCR tests also confirmed the results of bioinformatics analysis and showed that the expression of this gene in MS samples has a significant decrease in expression. These results make the hypothesis of diagnostic biomarkers

Table 5: Cellular signaling pathways of mRNA RPGR coding protein gene and other protein networks mentioned in Figure 3

Term	*P*	Adjusted *P* value	Odds Ratio
Cilium Assembly R-HSA-5617833	1.7176030321666058E-12	1.1336180012299598E-10	193.6731843575419
Organelle Biogenesis and Maintenance R-HSA-1852241	2.7104762766497688E-11	8.944571712944237E-10	128.77518656716418
RAS Processing R-HSA-9648002	0.010402987018789436	0.06655323472261897	110.95
Chaperone Mediated Autophagy R-HSA-9613829	0.010947779702855981	0.06655323472261897	105.10526315789474
MicroRNA (miRNA) Biogenesis R-HSA-203927	0.013124225781325726	0.06655323472261897	86.80869565217391
Cell Cycle, Mitotic R-HSA-69278	0.032108165359767765	0.08389858456518286	8.303689486031137
Selective Autophagy R-HSA-9663891	0.03305095755598113	0.08389858456518286	33.215
Signal Transduction R-HSA-162582	0.03717806377394846	0.08733841940403841	4.069890288500609
Gene Silencing by RNA R-HSA-211000	0.057835721217223385	0.09787583590607034	18.581308411214952
Autophagy R-HSA-9612973	0.06820614339416273	0.1088017183040322	15.639370078740157
MAPK1/MAPK3 Signaling R-HSA-5684996	0.14225898764381306	0.17387209600910486	7.142391304347826
Sensory Perception R-HSA-9709957	0.2912227105231511	0.33139136025048227	3.1502439024390245
Metabolism Of Lipids R-HSA-556833	0.33651972462527135	0.36410330861094936	2.634473324213406
Innate Immune System R-HSA-168249	0.44270309194825935	0.46378419156484313	1.8331721470019342
Gene Expression (Transcription) R-HSA-74160	0.5628625589346131	0.5804520139013197	1.2804558011049725
Immune System R-HSA-168256	0.675178955818414	0.6855663243694665	0.9292996910401647

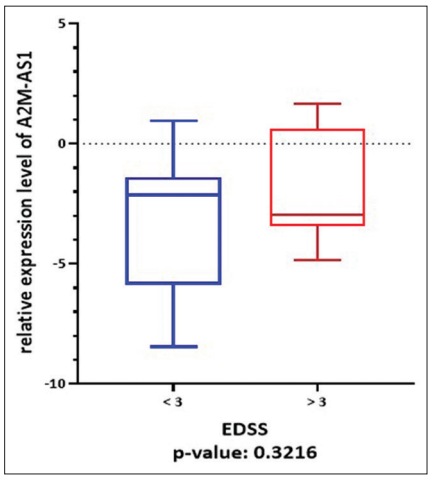

Figure 7: The results of examining the relationship between the expression of the studied gene and the pathophysiological characteristics of the patients. No correlation was found

of this gene stronger. Based on ROC statistical analysis performed on real-time PCR data, it was shown that A2M-AS1 lncRNA was identified as an excellent diagnostic biomarker. This result can strengthen the hypothesis that the reduction of A2M-AS1 expression is probably one of the possible reasons for subsequently increasing the risk of MS disease.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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