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The Potential Association Between microRNA 135-5P and p62 and Their Effect on NRF2 Pathway in Multiple Sclerosis

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Abstract

Background: Multiple Sclerosis (MS) is a prevalent non-traumatic disabling disease affecting young adults, characterized by complexity in its pathogenesis. Nuclear factor erythroid 2-Related Factor 2 (NRF2) serves as a crucial transcriptional regulator of anti-inflammatory and antioxidant enzymes, influenced by the ubiquitous protein p62. It acts as a scaffold directing substrates to autophagosomes. This study aims to explore the potential association between microRNA 135-5p and p62 and their impact on inflammation and oxidative stress through the NRF2 pathway in MS.

Methods: The study included 30 healthy controls and 60 MS patients (relapsing-remitting and secondary progressive). Real-time PCR was employed for the detection of Nrf2, p62, miRNA135-5P, and NF-κB in serum, while p53 levels were determined using ELISA.

Results: Nrf2 and p62 expression was significantly downregulated in the MS group compared to controls. Conversely, miRNA135-5P, NF-κB expression, and P53 levels were significantly elevated in the MS group.

Conclusion: This study reveals a potential association between miRNA 135-5p and p62, indicating their role in the pathogenesis of MS. Results suggest that miRNA 135-5p and p62 may influence inflammation and oxidative stress in MS through the NRF2 pathway, potentially mediated by NF-κB and p53.

Keywords: p62, Microrna135, Multiple sclerosis, NRF2.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory condition of the central nervous system (CNS), characterized by demyelination, neuronal degeneration, and concurrent axonal damage (1). It stands as the most prevalent nontraumatic disabling disease among young adults. The exact etiology remains elusive, with environmental, immune, and genetic factors contributing to its pathogenesis. Notably, increased oxidative stress and reactive oxygen species production have been implicated in MS development (2).

The endogenous antioxidant pathway, mediated by the transcription factor NRF2, defends cells against oxidative stress by elevating cytoprotective enzyme expression. NRF2 not only upregulates antioxidant gene expressions but also modulates mitochondrial function, biogenesis, and exerts anti-inflammatory effects (3). Normally bound to KEAP1 in the cytosol, NRF2 is regulated for degradation by proteasomes (4), making it a promising therapeutic target for neurodegenerative disorders characterized by neuroinflammation and mitochondrial dysfunction (5).

p62, a regulator of NRF2, acts as a scaffold protein and an autophagic degradation marker. Involved in autophagy, oxidative stress response, and cell signalling, p62 transports

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specific cargos to autophagosomes, sustaining NRF2 stabilization and translocation to the nucleus. NRF2 induces gene transcription, forming a positive feedback regulation loop with p62 (6).

The proinflammatory transcription factor NF-κB, a key regulator of genes in adaptive and innate immunity, is activated by oxidative stress. Significantly stimulated in chronic and acute inflammatory disorders, NF-κB plays a crucial role in host defense, triggering the upregulation of proinflammatory genes.

MicroRNAs (miRNAs), small non-coding molecules, regulate gene expression, influencing controlled cell processes such as proliferation, differentiation, and apoptosis. Altered miRNA expression is associated with various neurodegenerative disorders, making them potential diagnostic markers and therapeutic targets (8). miR-135-5p, identified as a negative regulator of p62, has been linked to the regulation of allergic inflammation, cellular interactions, and autophagic flux (10).

Apoptosis, a pivotal intracellular process for organismal homeostasis, plays a role in MS pathogenesis through the involvement of the p53 transcription factor. p53 contributes to processes like DNA repair, cell regulation, and apoptosis and is hypothesized to regulate neuronal death in disorders such as MS (11).

Materials and Methods

Patients and sample collection

The present research was performed in the Unit of Biochemistry and Molecular Biology (UBMB) in the Department of Medical Biochemistry and Molecular Biology in collaboration with the Department of Neurology, Faculty of Medicine, Cairo University. The local ethical committee in Kasr Al-Ainy Hospitals approved the study (Approval number: MD-361-2020).

The study was performed on 90 participants; 30 healthy controls with matching sex & age and 60 MS patients (relapsing-remitting & secondary progressive) recruited from the Neurology Department Outpatient Clinic, Kasr Al-Ainy Hospital, Faculty of Medicine, Cairo University. Informed consent was obtained from the participants. Inclusion criteria were patients of both sexes between 20 to 45 years and diagnosed with MS based on the revised McDonald criteria (12). Participants underwent complete medical history taking & clinical examination, including sex, disease course, positive family history, age, presentation mode (initial symptoms), and age of disease onset. Clinical examination, including general and neurological examination, was done.

Sample collection

Three millimeters of venous blood were collected from controls & patients and was left to clot and subsequently centrifuged for 5 min at 8000 x g to separate the serum that was preserved at -80 °C.

Gene Expression of NRF2, p62, and NF-κB in serum by RT-PCR

Qiagen kit (Qiagen, USA) was utilized for total RNA extraction from serum as per the manufacturer's instructions. RNA quality was evaluated utilizing a NanoDrop® 1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA). Then, complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific Baltics UAB, V.A. Graiciuno 8, LT-02241, Vilnius, Lithuania) following the manufacturer's utilizing Real time-qPCR and SYBR Premix Ex TaqTM II (Perfect Real Time, TaKaRa, Japan). The PCR reaction conditions were: 5 minutes at 95 °C, then 40 cycles at 95 °C for 15 seconds, and 60 °C for 60 seconds. To quantitatively examine the data, the RQ of each target gene is measured by normalizing against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase for (NRF2, p62 and NF-κB) using the calculation of the 2 -ΔΔCt. The relative quantitation was calculated using the Applied Biosystem software.

The values of the cycle threshold (Ct) were graphically obtained for both GAPDH as well as different target genes. All target genes' Ct values were first normalized to GAPDH in the same sample and expressed as ΔCt values. Afterward, ΔΔCt values were determined by the

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subtraction of the ΔCt values of the control samples from that of the treated samples, whereas $2^{-\Delta\Delta Ct}$ values were determined to represent different target gene amounts. The final values presented were expressed as ratios to control cells. The studied genes' primer sequence is displayed in Table 1

Gene expression of miRNA-135-5P by RT-PCR

miRNA was extracted from serum samples using mirvana kit, (Thermo Fisher, MA, USA). The TaqMan® Micro RNA Assays are designed to detect and accurately quantify mature miRNAs using Applied Bio systems real time PCR instruments. The process of synthesis of single-stranded cDNA from miRNA samples using the TaqMan® Micro RNA Reverse Transcription Kit, USA**,** using the U6 snRNA as an endogenous control for normalization. The comparative CT method (ΔΔCT) was utilized to determine the relative expression of the miRNA-135-5P gene within the patient and control groups. The studied gene' primer sequence is displayed in Table 1.

Table 1. Primer's sequence of studied genes.

Gene symbol	NCBI code		Primer sequence (5′- 3′)
NRF2	NM_001313904.1	F	AGCCCAGCACATCCAGTCAG
		R	TGCATGCAGTCATCAAAGTACAAAG
p62	NM_001142299.2	F	ATACGGGTGGGAATGTTGAG
		R	TTCTGGCATCTGTAGGGACTG
NFKB	XM_054350118.1	F	GCAGCACTACTTCTTGACCACC
		R	TCTGCTCCTGAGCATTGACGTC
GAPDH	NM_001289746.2	F	GCACCGTCAAGGCTGAGAAC
		R	TGGTGAAGACGCCAGTGGA
MiRNA 135-5P	NR_029677.1	F	CAGTGCAGGGTCCGAGGTAT
		R	CGTCGTATGGCTTTTTATTCC
U6	NR_004394	F	GCTTCGGCAGCACATATACTAAAAT
		R	CGCTTCACGAATTTGCGTGTCAT

*F: Forward primer, R: Reverse primer.

Statistical analysis

Data analysis, coding, and entry were done utilizing the 22nd version of the SPSS software. Data were summarized as SD and mean quantitative variables as well as relative frequencies (percentages) and frequencies (number of cases) for categorical variables. Comparisons between groups were made utilizing the unpaired t-test when comparing two groups and analysis of variance (ANOVA) when comparing more than two groups. For comparing categorical data, the Chi-square ($\chi 2$) test was performed. Correlations between quantitative variables were done utilizing the Pearson correlation coefficient (13).

Detection of P53/TP53 level by ELISA

ELISA was done based on the manufacturer's recommendations of Sun Long Biotech Co., LTD for the estimation of human p53 in serum (Catalog Number# SL 1323Hu).

Results

Demographic and disease characteristics of the study cohort

In our study, the MS patients included two types, the relapsing-remitting type (70%) & the secondary progressive type (30%)**.** The Mean ±SD of age was 32.8±6.9, and females represented a relatively larger proportion (75%) compared to males (25%) among MS patients. The mean age of onset of MS is 27.6±7.14 while the mean disease duration is 5.18±3.4. The most frequent first presentation of MS is motor presentation (46.7%), while the least is urinary symptom (1.7%) (Table 2).

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Table 2. Demographic and disease characteristics of the study cohort.

Variable	Patients (Total 60), N (%)
Sex (count/%)	
Females	45 (75%)
Males	15 (25%)
Age (Mean ±SD)	32.8±6.9
Age at onset (years)	18-42
Mean ±SD	27.6±7.14
Disease duration (years)	0-14
Mean ±SD	5.18±3.4
EDSS	
0.5 - 3.5	42 (70%)
4 – 6	18 (30%)
First presentation	
Ataxia	7 (11.7%)
motor	28 (46.7%)
sensory	12 (20%)
urinary	1 (1.7%)
visual	12 (20%)

Biochemical and molecular results

Gene expression of p62, NRF2, NF-κB, and miRNA135

Concerning the expression levels of NRF2 (transcriptional factor that regulates antioxidative response) & p62 (positive regulator of NRF2) genes, there was a substantial decline in their expression in the MS group than the control group (p-value < 0.001) (Fig. 1A).

Regarding the gene expression of NF-κB, there was a substantial elevation in its expression in MS than controls (P-value < 0.001) (Fig. 1B).

According to Fig. 1C, our results showed that miRNA 135-5P gene expression in the MS group was substantially elevated than controls (P value < 0.001).

P53 serum level Our results concerning the P53 level demonstrated a substantial elevation in the MS group compared to controls (P value < 0.001) (Fig. 1D).

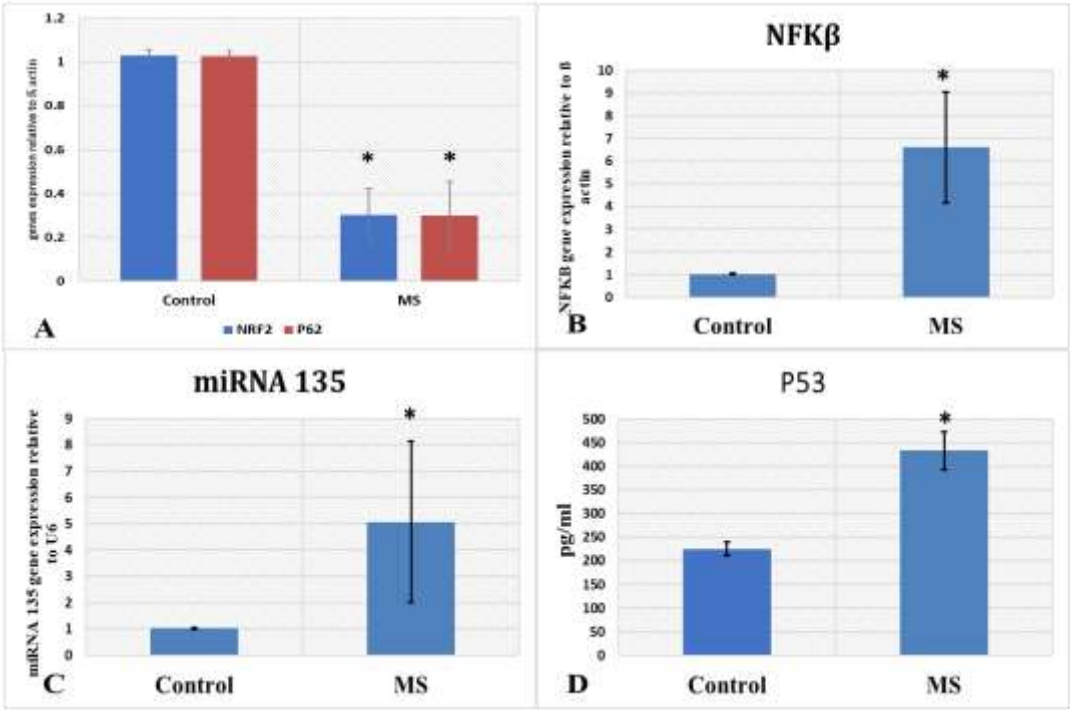

Fig. 1. A: comparison of NRF2 and p62 genes expression between studied group. **B**: comparison of NFKβ gene expression between studied groups. **C:** comparison of miRNA135 expression between studied groups. **D**: comparison of P53 level between studied groups. (*) Denotes significant difference compared to controls.

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Correlations

In our study, there was an inverse correlation between relapse and both NRF2 & p62 (r= -0.347, p-value =0.007) & (r= -0.343, p-value =0.007) respectively (Figs. 2A & B).

This study revealed a positive significant correlation between miRNA 135-5P and NF-κB (r= 0.289, p value =0.025) (Fig. 2C).

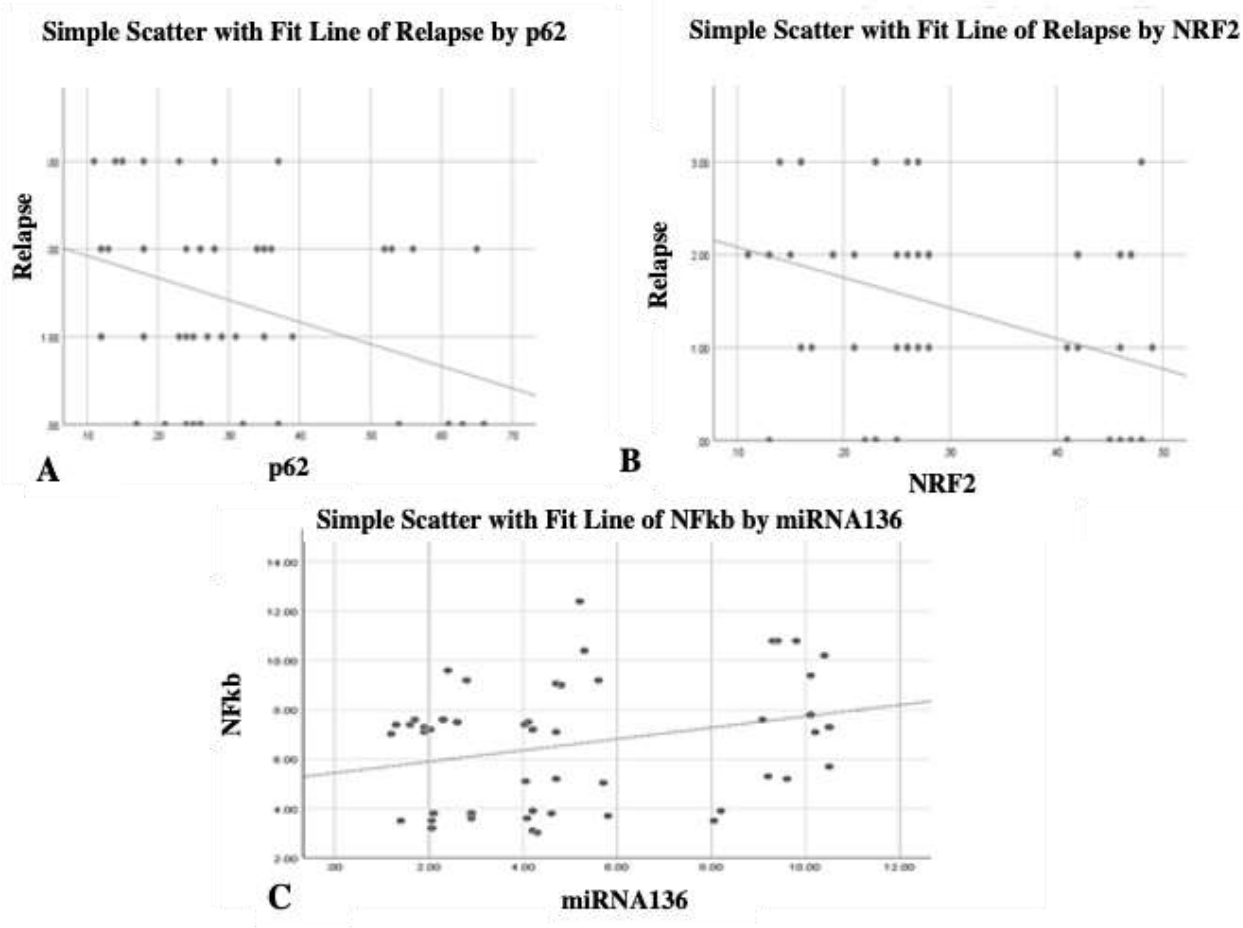

Fig. 2. A: Correlation between relapse and p62. **B:** Correlation between relapse and NRF2. **C:** Correlation between miRNA 135 and NFKB.

Discussion

This study highlights significant alterations in miRNA135-5p, NF-κB expression, P53 levels, NRF2, and p62 in multiple sclerosis (MS) patients compared to controls. Also, miRNA135-5p was positively correlated with NFkB, which highlights the possible role of miRNA135-5p in MS pathogenesis through its inflammatory effect.

The pathogenesis of multiple sclerosis (MS) remains unclear and complex, with various theories under consideration (14,15). Oxidative stress plays a crucial role in MS pathophysiology, as reactive oxygen species (ROS) are generated during cellular respiration and neurotransmission at concentrations deleterious to cell survival. In relation to NRF2, a transcription factor that regulates antioxidative responses, it was substantially diminished in the MS group compared to the controls. This finding aligns with the studies of (16,19), indicating a decrease in NRF2 expression in MS patients. Consequently, there is a reduction in antioxidant capacity, and mesenchymal stem cells (MSC) exhibit decreased secretion of GSTP and SOD1. This decrease may contribute to the pathogenic mechanisms observed in MS.

Consistent with our findings, a study by (20) reported that NRF2 induction through

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activators such as resveratrol and melatonin contribute to the regeneration of antioxidant responses and attenuation of inflammation in MS. Furthermore, another study demonstrated the beneficial effects of DMF (Dimethyl fumarate) on the clinical outcome in MS through the induction of the NRF2 pathway (21).

In addition to its antioxidant activity, NRF2 controls biogenesis and mitochondrial function, addressing symptoms associated with neuroinflammation and mitochondrial malfunction observed in various neurodegenerative disorders (3).

Furthermore, p62, a recently discovered ubiquitin-binding protein, is considered a positive regulator of NRF2 (17). In our study, p62 gene expression exhibited a significant decline in the MS group compared to controls. This finding is consistent with a study by (22), which demonstrated that the oxidative effects of diabetes in testicular tissues might be mediated by reduced p62 expression.

Another study (23) supports our results, reporting diminished p62 expression in Alzheimer's Disease (AD), and loss of p62 function leading to neurodegeneration. This finding aligns with another study indicating reduced levels of p62 in mice's spinal cord at all stages of experimental autoimmune encephalomyelitis (EAE) (24). Additionally, P62 negatively regulates the inflammasome pathway through the degradation of ubiquitinated inflammasome proteins via autophagy (25). P62 protects against neuroinflammation and neuronal death by eliminating toxic tau species in a mouse model brain (26).

In contrast to our study, in a spinal muscular atrophy (SMA) mouse model, p62 protein levels were elevated in spinal cord lysates, indicating autophagy dysregulation (27).

The current study demonstrated a negative correlation between relapse and p62 and NRF2 levels. This result suggests using them as a predictor of relapse in MS patients.

Due to the crosstalk between anti-inflammatory and antioxidant pathways, several of NRF2's mitochondrial and anti-inflammatory actions are deemed secondary to its antioxidant actions, as NF-κB is upregulated via oxidative stress, can be blocked by induction of the NRF2-dependent antioxidant target genes. Additionally, Nrf2 inhibits NF-κB nuclear translocation (30). Evidently, NF-κB and Nrf2 interact in neurodegenerative disorders, where the elevation in NF-κB exacerbates neuroinflammation, whereas elevated Nrf2 provides neuroprotection (31).

Regarding NF-κB, the proinflammatory transcription factor, our observations indicate a substantial upregulation of NF-κB expression in MS cases compared to controls. This aligns with findings from (28,29), which demonstrated NF-κB activation in various cell types within MS and CNS cases, including neurons, oligodendrocytes, astrocytes, microglia/macrophages, and T cells (15). NF-κB serves as a significant gene regulator in both adaptive and innate immunity, displaying heightened activity in chronic and acute inflammatory conditions and being responsible for the upregulation of proinflammatory genes (7).

Another study by (28) supports our findings, reporting that aberrant NF-κB activation contributes to MS by increasing proinflammatory cytokines, leading to inflammatory demyelination of the CNS.

Concerning miRNAs, they play a crucial role in regulating gene expression, controlling cell proliferation, differentiation, and apoptosis (8). Altered miRNA expression has been linked to various neurodegenerative disorders, including miRNA-135-5p (18). To our knowledge, miRNA-135-5p expression had not been determined in MS before this study. Our findings reveal a substantial elevation of miRNA-135-5p in MS patients compared to controls. This aligns with the results from (33), who discovered that high expression of miRNA-135-5p contributes to epilepsy-induced nerve cell apoptosis, while a miRNA-135-5p inhibitor protects nerve cells against epilepsy-induced apoptosis, presenting a novel strategy for treating seizure-induced neural damage. MiRNA-135-5p is expressed

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in various tissues, including the brain, cerebellum, ovary, prostate, and others (32).

MiRNA-135-5p exhibits multiple functions in both neoplastic and non-neoplastic conditions and holds potential applications as a marker for both. Our results are consistent with another study demonstrating that the upregulation of miRNA-135-5P plays a crucial anticancer role in ovarian cancer by significantly reducing cell proliferation while promoting cell apoptosis (34). Additionally, (35) demonstrated a high expression level of miRNA-135-5P associated with disease activity in rheumatoid arthritis patients.

A prior study discovered that miRNA-135-5p may contribute to depression by reducing SIRT1 expression. Inhibiting miRNA-135-5p alleviates neuronal apoptosis, substantially reduces the expression levels of cleaved caspase-3, and decreases inflammatory responses by upregulating the expression of SIRT1. Consequently, it contributes to the antidepressant effect of the miRNA-135-5p inhibitor (36).

In contrast to our study, the inhibition of miRNA-135-5p induced memory impairment and synaptic disorders in Alzheimer's disease (AD), suggesting a potential therapeutic role of miRNA-135-5p in AD (37). Kim et al. found that the miRNA-135-5p-p62 axis regulates allergic inflammation mediated by extracellular vesicles in rat basophilic leukemia (RBL2H3) cells (10). They observed that miRNA-135-5p diminished the p62 expression level, and thus, they reported that miRNA-135-5p acts as a negative regulator of p62. These results coincide with our work, as we hypothesize that miRNA-135-5p may act as a negative regulator of p62 in MS.

Our findings also revealed a positive association between miRNA-135-5p and NF-κB, aligning with (38), who illustrated that miR-135-5p induction is mediated by NF-κB's activation and inflammatory responses in gastric cancer cells.

P53, a transcription factor involved in apoptosis (39), acts as a tumor inhibitor, limiting cell proliferation, stimulating apoptosis, and inducing cell cycle arrest in response to cellular stresses (40). In this study, the p53 level was considerably elevated in MS cases compared to controls. This finding aligns with Valiullina et al., who observed elevated expression levels of p53 in MS patients compared to healthy controls, indicating induction of apoptosis and neuronal death (11).

Wild-type p53 functions both as an antioxidant and pro-oxidant factor, regulating intracellular ROS levels. Under basal stress levels, basal p53 reduces ROS production by inducing several antioxidant gene expressions. However, under severe stress, p53 is elevated, leading to the induction of pro-oxidant and proapoptotic genes (41).

The crosstalk between p53 and Nrf2 is crucial for maintaining cellular homeostasis. In prior research, Nrf2 was shown to be required for the basal transcription of E3 ubiquitin ligase MDM2, which regulates p53 by poly-ubiquitinating it for proteasomal degradation, ultimately promoting p53 degradation (42). This is consistent with our results, indicating that a low expression level of NRF2 is associated with elevated P53 levels.

In conclusion, our study revealed a significant increase in miRNA-135-5p, NF-κB expression, and P53 levels, while there is a substantial decline in NRF2 and p62 expression in MS patients compared to controls. These findings suggest the potential use of miRNA-135-5p, NF-κB, and P53 as therapeutic targets and diagnostic markers in MS.

Ethical approval

All measures were carried out following the ethical considerations of the Research Committee of Kasr Al-Ainy, Cairo University Hospitals, Approval number: MD-361-2020. All subjects provided informed consent prior to data collection and following the explanation of research objectives.

Conflicts of interest

All authors declare no conflicts of interest.

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