

## Impact of miR-155 Gene Polymorphism (rs767649 A>T) and miR-155 Gene Expression on Susceptibility to Multiple Sclerosis

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### ABSTRACT

**Background:** Micro RNA155 (miR-155) was identified as an essential determinant in immunological responses, and its genetic variants have increasing attention due to their ability to modulate its expression and potentially influence the susceptibility to autoimmune illnesses including rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus, etc.

**Objectives:** To examine the impact of miR-155 gene polymorphism (rs767649A>T) and miR-155 gene expression and their association with multiple sclerosis (MS) in a sample of Iraqi patients.

**Materials and methods:** A total of 75 blood specimens were obtained from individuals diagnosed with MS. While an additional 75 blood specimens were collected from evidently healthy participants serving as a control group, with an age ranged 20-71 years. miR-155 gene polymorphism (rs767649 A>T) was determined utilizing Tetra-ARMS Polymerase Chain Reaction (Tetra-ARMS PCR) and miR-155 expression was evaluated using Real-time Polymerase Chain Reaction (RT-PCR).

**Results:** The females experienced MS at a higher rate (69.33%) compared to the males. Furthermore, the age group 30-39 years showed a greater susceptibility to the disease (54.67%). The analysis of miR-155 (rs767649 A>T) SNP in MS patients indicated that 7 (9.34%) had the wild genotype (AA), 31 (41.33%) had the heterogeneous genotype (AT), and 37 (49.33%) had the mutant genotype (TT). These differences were statistically significant (P-value = 0.040). A allele frequency was 45 (0.3) (OR: 0.25; CI: 0.15-0.41) and T allele frequency was 105 (0.7) (OR: 3.91; CI: 2.42-6.33) in MS patients. While analysis of miR-155 gene expression demonstrated a significant increase in the patient group ( $1.82 \pm 0.25$  fold) compared to the healthy control group ( $0.33 \pm 0.13$  fold). The relationship between miR-155 gene expression and miR-155 genotypes in MS patients, revealed a notable elevation in miR-155 gene expression at the TT genotype ( $3.15 \pm 0.73$  fold), followed by TA genotype ( $1.29 \pm 0.65$  fold) and finally AA genotype ( $0.37 \pm 0.19$  fold) with highly statistically significant difference (P-value = 0.001).

**Conclusion:** There was a significant positive correlation between miR-155 (rs767649 A>T) genotypes and miR-155 expression and susceptibility to MS in Iraqi patients. These findings suggests that miR-155 may hold potential as a diagnostic and therapeutic marker for the disease.

**Keywords:** Multiple Sclerosis; rs767649 A>T; Genotyping; miR-155; Expression.

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### INTRODUCTION

**M**ultiple sclerosis (MS) is a chronic autoimmune disorder affecting the central nervous system (CNS) identified by inflammation, demyelination, and neurodegeneration, many epigenetic,

genetic, and environmental factors influence MS development [1, 2].

Various investigations are supporting a vital capacities of small non-coding micro RNAs (miRNAs) as governing controllers of biological operations linked to the pathophysiology of diverse disorders, including autoimmune and neurodegenerative diseases [3].

The important criterion of miRNAs, it's ease to obtaining as they presented in body fluids, which eliminates the need

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for invasive isolation from cells or tissues. Existing data indicates miR-155 as a potent inflammation stimulator and a crucial contributor to autoimmune disorders due to its ability to enhance myeloid cell polarizing towards proinflammatory traits [4].

A multifunctional miR-155 exhibits the ability to regulate genes involved in immune response including TNF- $\alpha$  and NF- $\kappa$ B transcripts, which are essential in the neuroinflammation onset, and involved in CNS's resident cells production of proinflammatory cytokines in addition to activation of glial cells, a processes commonly observed in various neurodegenerative disorders. miR-155 was shown to be overexpressed in different MS samples, encompassing resident cells, active lesions of the brain, and blood [5].

The rs767649 T>A SNP found in the miR-155 promoter region, was observed in various autoimmune disorders, including rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus, and MS [6, 7]. Consequently, it is reasonable to anticipate that this polymorphism may contribute to alterations in miR-155 expression and subsequently affect the susceptibility to MS.

Explore the potential contribution of miR-155 expression and rs767649 T>A polymorphism in MS may offer novel indicators of neurodegeneration that can be used in the diagnosis or management of MS, in addition it could be useful for understanding the role of these agents in MS pathogenesis. Therefore, this study aimed to detect the genotyping of rs767649 T>A and miR-155 expression, in addition to, assessing the effect of rs767649 T>A genotypes on miR-155 gene expression in MS patients.

## MATERIALS AND METHODS

Our study was conducted in the laboratories of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad, Iraq, through the period from May 2022 to the end of April 2023. The research ethics committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/University of Baghdad was reviewed and approved the study protocols, subject information, and consent form, according to document number EC/1305A dated May 15, 2022.

The Kelsey equation in Epi Info version 7.2 was used to calculate a sample size of 56 for patients and 56 for controls with a 99% confidence interval. However, we enrolled 75 patients during the specified duration of the study. Male or female patients of all ages who were diagnosed with MS and followed by a neurologist of MS clinic in Baghdad Teaching Hospital at Medical City, Saad AlWatri Hospital for Neurological Sciences in Baghdad, and Al-Yarmouk Teaching Hospital, as well as, age- and gender-matched 75 healthy volunteers as a control group were included in this study. Females of childbearing potential must have a negative pregnancy test. All participants with chronic disease of the immune system other than MS, malignancy, active systemic bacterial, viral or fungal infections or any medically unstable condition were excluded to avoid the possibility of the miR-155 expression and rs767649 T>A interfering with these conditions. All participants were signed an information/consent document after being informed of the purpose and advantages of the study. Participant's information includes sex, age, and if they suffer from any disease other than MS.

Three milliliters of blood samples were obtained from all participants and transferred into EDTA anticoagulant tubes, mixed gently, and stored at -20°C until it was subjected

to DNA extraction using the DNA purification kit (Geneaid/Taiwan) for the detection of (rs767649 T>A) polymorphism using Tetra-ARMS Polymerase Chain Reaction (Tetra-ARMS PCR) method. The primers specified in Table 1 were used to amplify the DNA fragments while the PCR reaction consisted of initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation (95°C/45 sec), annealing (56°C/1min.), and extension (72°C/40 sec.), followed by final extension step (72°C/10 min) and hold step (4°C/ $\infty$ ). The PCR products (841bp, 485bp, 404bp) were separated by electrophoresis on a 2.5% agarose gel.

Immediately after collection, 250  $\mu$ l of blood sample was transferred from anticoagulant tubes, mixed with 750  $\mu$ l of Trizol and stored at -20°C for RNA purification using Promega kit /USA. The concentration and purity of DNA and RNA were determined via Nanodrop (Bioneer/Korea).

Detection of miR-155 expression was performed via a two-step RT-qPCR approach. The first step included the conversion of RNA to complementary DNA (cDNA) using an AddScript Reverse Transcriptase kit (addbio, Korea) according to protocol outlined in Table 2. Subsequently, the second step was carried out by employing the specific primers obtained from Macrogen (Korea) as detailed in Table 3. The RT-qPCR reaction was performed via Mic qPCR Cycle (Bio Molecular System/ Australia) which was programmed with a thermo cycling protocol as follows: 1 cycle of initial denaturation at 95°C, 45 cycles of denaturation at 95°C, and annealing at 60°C, while the temperature of melting curve was 60-95°C.

The results were presented as mean  $\pm$  S.E., and T-test were employed to determine the significance of the comparison between two samples. On the other hand, the significance between means of more than two samples was evaluated using the least significant difference (LSD) test (Analysis of Variation-ANOVA) while the Pearson Chi-square test ( $\chi^2$  test) (IBM SPSS- version 28) was used to determine the significance of different percentages. Whenever a P-value < 0.05 was considered statistically significant.

## RESULTS

The demographic findings demonstrated that females (n = 52, 69.33%) were more susceptible to MS than males (n = 23, 30.67%) with significant differences (P-value = 0.001), as well as according to age at diagnosis, the results showed that the age group 30-39 years had highest incidence rate (n = 41, 54.67%). There was a highly substantial difference (P-value = 0.0001) as shown in Table 4.

The result of miR-155 (rs767649 T>A) genotyping revealed that the T-ARMS-PCR product was divided into three bands (841bp, 485bp, 404bp): 841 bp represented outer PCR which served as a DNA template for the inner allele A (485bp) and the allele T (404bp). The wild genotype (AA) had only two bands of 841 and 485 bp; the heterozygous genotype (AT) had three bands of 841, 485bp, and 404bp; and homogenotype "mutant" (TT) had two bands of 841 and 404 bp (Figure 1).

The wild genotype (AA) was observed in a 7 patients which represented a percentage of 9.34%, when compared to the control group (n = 29, 38.67%), with a highly statistically significant difference (P-value = 0.004). The heterogeneous AT genotype was frequented 31(41.33%) times in patient group, and 36 (48%) in the control group, with significant differences (P-value = 0.037). Also the genotype TT was repeated 37 times in the patient group representing a percentage of 49.33, and was 10 times in the control group represented a percent-

**Table 1.** The miR-155 T>A (rs767649) primer sequences for Tetra-ARMS PCR.

Primers	Sequences 5' → 3'	Annealing temp.	Product size	Reference
Outer F	TGTCTATGACCACTAATTCCCAC	56°C	841 bp A 485 bp T 404 bp	[8]
Outer R	AAATTTGGGTTAAATGATGTCAC			
Inner F	ATATAACACATTATCAAAAACACCGT			
Inner R	ATTAGAGCACTCAGAAAAGCGT			

**Table 2.** PCR program for cDNA conversion.

Steps	°C
Priming	25 for 10 min
Reverse transcription	50 for 60 min
RT inactivation	80 for 5 min
Hold	12 ∞

**Table 3.** Primers of miR-155 and U6 reference gene with their sequences.

Primer name	Sequences 5' → 3'	Reference name
miR-155-F	GTGGGTTAATGCTAATCGTGAT	This study
miR-155-R	GTTGGGTGTCAGTTTGTCAAAT	
U6-F	CTCGCTTCGGCAGCAC	[9]
U6-R	AACGCTTACGAATTTGCGT	

age of 13.33, with a highly substantial difference (P-value = 0.006). A allele frequency was 45 (0.30%) in the patients group and 94 (0.626%) in the control group, with an odds ratio of (OR: 0.25) and a confidence interval (CI: 0.15-0.41) while the T allele frequented 105 (0.70) in the patients group, as well as, 56 (0.373) (OR: 3.91; CI: 2.42-6.33) in the control group with a highly substantial difference (P-value = 0.009) as shown in Table 5.

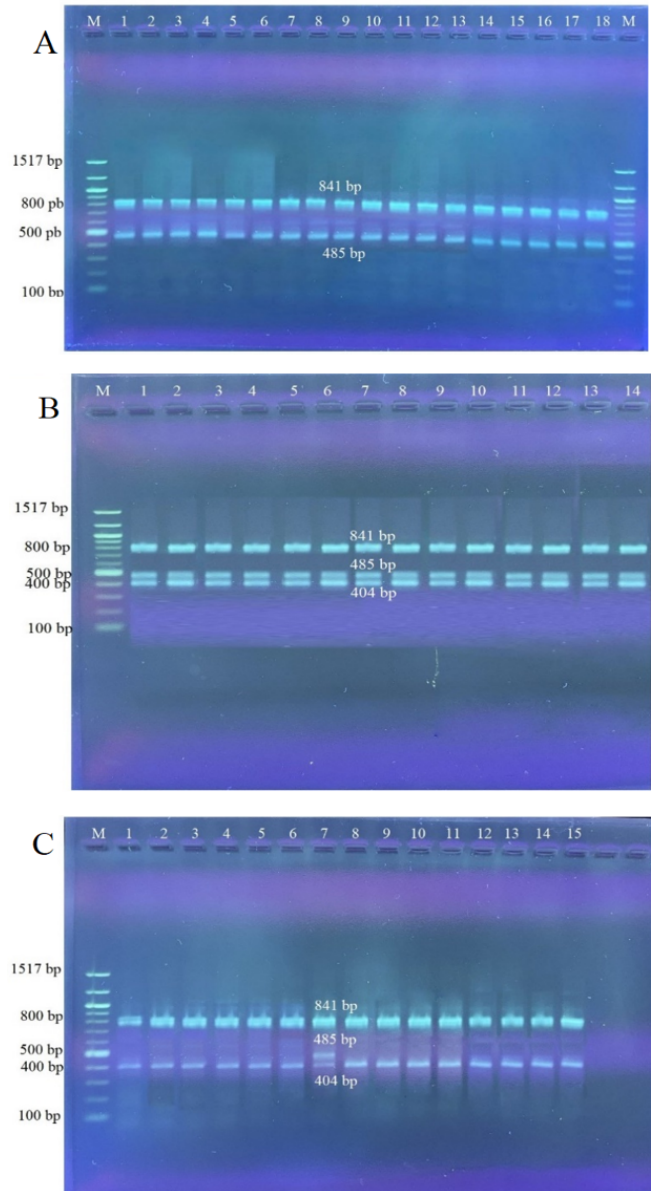
The miR-155 gene expression analysis revealed a significant increase in the folding mean found in the patient group (1.82 ± 0.25 fold) compared to the control (0.33 ± 0.13 fold), with significant differences (P-value = 0.022) as elucidated in Table 6.

The result revealed a substantial increase in miR-155 expression of 3.15 ± 0.73 fold in patients with TT genotype, followed by 1.29 ± 0.65 fold in those with AT genotype, and finally 0.37 ± 0.19 fold in those with AA genotype, with significant variations (P-value = 0.001) as shown in Table 7.

**DISCUSSION**

During the past decade, micro RNAs have been identified as regulatory key of inflammation and demyelination processes associated with MS, playing a crucial role in the pathophysiology of MS via regulating both innate and adaptive immune responses [3]. Our study’s main finding was the significant correlation between miR-155 (rs767649 A>T) genotypes and miR-155 expression with susceptibility to MS in Iraqi patients.

The results revealed that females were more susceptible to MS compared to males, several physiological and biological



**Figure 1.** The result of miR-155 (rs767649 T>A) SNP genotyping. A: AA genotype, B: AT genotype, C: 1-6 and 8-15 are TT genotype, 7 is an AT genotype and M is the marker.

factors of females including menstruation, pregnancy, breast-feeding, and others were found to cause changes in hormones levels leading to impact the immune system [10, 11]. On the other hand certain studies propose that the presence of two

**Table 4.** The age and sex distribution of the multiple sclerosis patients.

Sex No. (%)		Total	$\chi^2$	P-value		
Male	Female					
23 (30.67)	52 (69.33)	75 (100)	11.21	0.001		
Age group (years) No. (%)		Total	$\chi^2$	P-value		
20-29	30-39				40-49	$\geq 50$
12 (16)	41 (54.67)	15 (20)	7 (9.33)	75 (100)	36.94	0.0001

**Table 5.** Genotypes distribution and allele frequency of miR-155 (rs767649 A>T) in the study groups †.

Genotypes	Patients No.(%)	Control No.(%)	$\chi^2$	P-value	O.R.(C.I.)	P-value
Wild: AA	7 (9.34)c	29 (38.67) b	Ref.	-	0.16 (0.06-0.40)	0.004**
heterogeneous: AT	31 (41.33) b	36 (48) a	4.23	0.037*	1.76 (0.40-1.45)	0.037**
mutant: TT	37 (49.33) a	10 (13.33) c	28.7	0.0001**	6.32 (2.82-14.15)	0.006**
Total	75 (100)	75 (100)				
$\chi^2$	4.198	4.02				
P-value	0.040*	0.047*				
Alleles	Frequency		$\chi^2$	P-value	O.R.(C.I.)	P-value
A	45 (0.30)	94 (0.626)	Ref.	-	0.25 (0.15-0.41)	0.002**
T	105 (0.70)	56 (0.373)	32.18	0.0001**	3.91 (2.42-6.33)	0.007**
$\chi^2$	6.018	4.050				
P-value	0.009**	0.032*				

†  $\chi^2$ =Chi-Square, -\*\* (P-value  $\leq$  0.01), \* (P-value  $\leq$  0.05), (a, b, and c) different letters in same column mean differed significantly.

**Table 6.** Comparison of the expression of miRNA 155 in MS patients and control groups.

Groups	Mean $\pm$ SE					
	CT of U6 Ref. Gene	CT	Delta CT	Delta Delta CT	Folding	
Patients	18.02	28.00	9.99	0.04	1.82 $\pm$ 0.25	
Control	15.30	28.40	13.04	2.96	0.33 $\pm$ 0.13	
T-test					1.67	
P-value					0.022*	

\* P-value  $\leq$  0.05

**Table 7.** Relationship between miR-155 (rs767649 A>T) genotype and miR-155 gene expression †.

Genotype	Mean $\pm$ SE of miR155 gene expression (Folding)		$\chi^2$	P-value
	Patients	Control		
AA	0.37 $\pm$ 0.19	0.55 $\pm$ 0.37	1.34	0.69 <sup>NS</sup>
AT	1.29 $\pm$ 0.65	0.60 $\pm$ 0.29	5.23	0.044*
TT	3.15 $\pm$ 0.73	0.78 $\pm$ 0.44	9.21	0.010*
LSD value	1.43	0.41		
P-value	0.001**	0.001**		

†  $\chi^2$ =Chi-Square, \*\* (P-value  $\leq$  0.01), \* (P-value  $\leq$  0.05), NS: Non-Significant.

X chromosomes in females plays a crucial role in the production of myelin proteo-lipid protein (PLP), the predominant protein found in myelin of central nervous system, which is encoded by genes on the X chromosome and is a likely target of immune attack in MS. Also X chromosome may affect how its genes are expressed, leading to down regulating genes that provide protect versus autoimmunity, or up regulate genes

that increase the susceptibility of autoimmune diseases [12]. The present findings were corroborated by a study conducted by Al-Hamadani, [13] and Hassoun *et al.* [14] who reported a greater prevalence of MS in females than in males. Similarly, the current results are aligned with those of Hammood *et al.* [15] who observed notable disparities in the incidence of MS between males (40%) and females (60%), as well as variations

in the severity of the disease.

It can be noticed that MS can attack individuals at any age. The result indicated that the age group of 30-39 years was more susceptible to the disease, while patients over the age of 50 were found to be less prone to developing this disease. Also the younger ones were less likely to be affected because they had a robust immune system. Although certain studies had indicated instances of MS in younger age groups, when reported that occurrence of the MS disease in pediatric age [15]. The reasons of that may be due to the modern lifestyle and nutrition based on fast food without paying attention to the basic elements of food in addition to sitting long in front of the television, computer, and phone without going out to fresh air and exercising. The current result aligns with the study of Abdulhameed and Mohammed [16] who likewise observed a high prevalence of MS at thirty years of age. Nevertheless, some of the previous results contradicted the current study due to the interaction of many factors with the pathogenesis of MS as a study by Khademi [17] who found no significant differences between the ages of thirty-two and thirty-five.

Genotyping and allele frequency of rs767649 A>T SNP showed that this SNP was a risk factor for MS in Iraqi patients, where the mutant T allele and TT genotype were identified as risk factors for MS susceptibility. The current results consistent with previous research investigating the association between rs767649 A>T and susceptibility to MS, as well as to other autoimmune diseases [18, 19]. The TT genotype and T allele of rs767649A>T were correlated with elevated expression levels of miR-155 in non-small cell lung cancer and hepatocellular carcinoma, in addition to influencing the miR-155 up regulation in many processes connected to the pathogenesis of MS [20, 21]. Based on that, the findings of the current investigation could be comprehend through that, rs767649A>T mutant allele may cause elevation in miR-155 expression, consequently, leading to increase susceptibility to MS.

In the current investigation, MS patients were found to have increased expression of miR-155, this finding was in accordance with other studies [22–25]. Furthermore, the study of Lyons [26] demonstrated the significant involvement of miR-155 in promoting chronic inflammation in the central nervous system and confirmed its pathogenic role in macrophages during experimental autoimmune encephalomyelitis (EAE). This epigenetic factor is an important player in intricate mechanisms participating in the MS pathophysiology, encompassing demyelination, permeability of blood-brain barrier (BBB), neuropathic pain, and neuroinflammation. Overexpression of miR-155 plays a role in the activation of macrophages by down regulating the expression of CD47 in oligodendrocytes and astrocytes, leading to phagocytosis of myelin by macrophages [27], as well as, regulation of MS risk genes, PIK3CA and PIK3R1, which encode for p85- $\alpha$  and p110- $\alpha$  proteins, both members of the phosphoinositide 3-kinase (PI3K) family. Anomalies in PI3K participates in EAE pathogenesis, neurological and immunological dysfunctions, and demyelination in MS [28].

Our results demonstrated that the mutant T allele and TT genotype lead to significant miR-155 overexpression in MS patients. Based on ENCODE data, the functional rs767649 A>T has been present in the putative enhancer element of A549 cell line. Genetic variants within the regulatory regions of miRNAs have garnered attention due to their ability to alter miRNA expression and potentially influence the risk of various diseases [29]. The mutant T allele of rs767649 A>T

might increase the risk of MS disease via enhancing the expression of miR-155 gene. This hypothesis is supported by the results of Xie et al. [21] when temporary transfecting the luciferase reporter plasmids (with T or A allele of rs767649 A>T) and pRL-SV40 plasmids in the A549 cell line, it revealed that the reporter gene with the rs767649 T allele had substantially luciferase up regulation (P-value = 0.01) than the gene with the A allele, concluding that the rs767649 A>T increases the transcriptional activity of the miR-155 gene through binding with NF- $\kappa$ B, which acting as a transcriptional activator.

The following limitations should be considered; firstly, the possible mechanisms of how miR-155 affect MS should be evaluated, and secondly, the present study cannot rule out that other SNPs located in miR-155 could be linked to MS.

## CONCLUSION

The miR-155 rs767649A>T polymorphism was considered a risk agent for the MS for first time in Iraqi patients where mutant TT genotype and T allele were related to a heightened risk of MS. The miR-155 expression in the blood of MS patients was up regulated in comparison with healthy control, whilst the TT genotype of rs767649A>T SNP was significantly related to miR-155 overexpression and a correlation was observed between the overexpression of miR-155 in MS patients and the presence of the mutant T allele in heterozygous AT and mutant TT genotypes. Overall, the results of this study reported that both miR-155 and its functional (rs767649 A>T) SNP as potential elements for diagnosing or managing of MS.

## ETHICAL DECLARATIONS

### Acknowledgments

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### Ethics Approval and Consent to Participate

Written approval was obtained from the Ethical Approval Committee of the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies/University of Baghdad (EC/ 1305A on May 15, 2022). Informed consent was obtained from each participant.

### Consent for Publication

No personal data included.

### Availability of Data and Material

The datasets produced and/or analysed during the present study can be obtained from the corresponding author upon reasonable request.

### Competing Interests

The authors declare that there is no conflict of interest.

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## Authors' Contributions

Both authors have made significant, direct, and intellectual contributions to the work and have approved it for publication.

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