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Research Paper: The Association Between *ANKRD55* rs6859219 SNP and the Risk of Multiple Sclerosis in Iranian Patients

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ABSTRACT

Background: Multiple Sclerosis (MS) is a neuroinflammatory autoimmune disease of the central nervous system. *ANKRD55* rs6859219 Single Nucleotide Polymorphisms (SNPs) are reported as a potential novel MS risk gene, but the biological reasons underlying this correlation are still mysterious.

Objectives: This study aimed to investigate the potential association of *ANKRD55* rs6859219 SNP with the risk of MS in Iranian patients.

Materials & Methods: Blood samples were taken from 80 patients and healthy individuals. Afterward, the effects of some related-risk factors, such as age, gender, smoking status, drug allergy, and exposure to chemicals (such as detergents, cleansing chemical, acids, etc., in their common work area or their daily home exposure) were measured on the incidence of Relapsing-Remitting MS (RRMS) patients. Polymerase Chain Reaction (PCR) was performed, and the PCR products were genotyped using the Sanger sequencing system.

Results: The GT genotype of *ANKRD55* rs6859219 SNP more increased in MS patients than healthy subjects, and a significant association was found between MS and the rs6859219 SNP in the single-variable association analysis (P=0.04). In the additive model, our findings indicated that the rs6859219 SNP increased the risk of MS. Additionally, the results of all crude models, additive, recessive, dominant, and overdominant, were significantly different in participants with MS compared to the controls.

Conclusion: Eventually, the GT genotype of *ANKRD55* rs6859219 SNP significantly increased the risk of MS compared to the TT genotype.

Keywords: Multiple sclerosis, *ANKRD55* protein, Polymorphism, Single nucleotide

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Caspian Journal of Neurological Sciences

Highlights

- An association was found between the risk of MS and *ANKRD55* rs6859219 SNP.
- The GT genotype of *ANKRD55* rs6859219 SNP significantly increased the risk of MS compared to the TT genotype.

Introduction

Multiple Sclerosis (MS) is an immune-mediated auto-inflammatory disease of the Central Nervous System (CNS) in which the myelin sheaths of the neuronal cells are damaged in the brain and spinal cord [1]. MS has a relatively high incidence among young and middle-aged people and is commonly seen in four clinical forms: PRMS, PPMS, SPMS, and RRMS (Relapsing-Remitting MS). Among these, RRMS includes approximately 85% of cases [2]. Etiopathology of MS is somewhat uncertain, but it is believed that the complex interactions between genetic and environmental factors, including oxidative stress, viral infections, smoking status, exposure to chemicals, etc., affect the disease [3-5]. Despite some disease-modifying treatments for MS, there is no specific treatment strategy for all MS patients until now. Therefore, the study of factors involved in disease development, in particular genetic factors, could be helpful in effectively treating the disease. In the last decade, many genes associated with MS have been successfully identified, some of which may potentially contribute to the disease [6, 7]. However, several experiments are required to confirm the association of these genes with MS in detail. Individually, it has been determined that the genetic diversity of some of these genes is a potential risk factor for the functioning of CNS in humans and may be responsible for many MS-related disorders.

Investigating Single Nucleotide Polymorphisms (SNPs) of MS-related genes is a critical approach to discovering the disease's mechanism [8]. A maximum of 90% of the genetic varieties in the human genome is due to SNPs. The Ankyrin Repeat Domain-55 (*ANKRD55*) gene has been primarily involved in rheumatic and autoimmune diseases, and it has been recently identified as a novel candidate gene for the risk of MS [9, 10]. *ANKRD55* is located on the chromosomal region 5q11.2, has 14 exons, and encodes 614 amino acids, and rs6859219 is located in intron 7 of the gene [11]. *ANKRD55* encodes the "ankyrin repeat domain-containing protein-55 (*ANKRD55*)", whose biological function is almost unknown, but given that ankyrin repeat domains organize one of the most common protein-protein communication platforms, it is likely to function in coordination with other proteins [12]. *ANKRD55* is a hazardous gene for various autoimmune diseases such as rheumatoid arthritis [10], type 2 diabetes [13], Crohn's disease [14], celiac disease [15], multiple sclerosis, Interstitial Lung Disease (ILD), and Dermatomyositis/Polymyositis (DM-PM) [16-18]. In addition to *ANKRD55* variations, SNPs near *ANKRD55* are associated with changes in the N-glycosylation of IgG, which is altered in some inflammatory conditions such as rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune disorders [19]. In this regard, defective N-glycosylation has also been implicated in MS [20, 21].

Some previous studies indicate a significant association between polymorphism rs6859219 in the *ANKRD55* gene and the risk of MS [9, 10, 20]. Our study aimed to examine this relationship in Iranian MS patients. Moreover, we intended to determine the association of *ANKRD55* with the allele and genotype distribution of rs6859219 and assess factors such as smoking status, drug allergy, exposure to chemicals, age, and gender. The present study is the first research in Iran investigating the relationship between the *ANKRD55* rs6859219 SNP and the risk of MS.

Materials and Methods

General characteristics of the population and sample collection

In this cross-sectional case-control study, we included 40 RRMS patients and 40 healthy participants as controls. MS cases and control groups were age- and gender-matched. The genotypic frequencies in both controls and patients were adjusted to the proportions expected by the Hardy-Weinberg Equilibrium (HWE) via SPSS, version 24 (IBM Corp., Armonk, NY, USA), demonstrating that the control group was representative and in stable condition.

After obtaining written informed consent from patients and healthy subjects according to the ethical guidelines in the Iranian Biological Resource Center (IBRC), we

Table 1. General properties of the primers and their sequences

Primer	Tm (ºC)	GC%	Product Length (bp)	Primer Length (bp)	Primer Sequence
Forward	62.07	60.00	393	20	TGGGCACCTGGGACTTTCAG
Reverse	60.55	50.00	393	22	GTAGAAAGTGCCTGCTTCTGGT

collected some data such as age and gender and related data from their medical files. We also collected information about drug allergy, smoking status, and exposure to harmful environmental and chemical agents by employing a questionnaire or conducting an interview. Ethylenediaminetetraacetic Acid (EDTA)-peripheral blood sampling from healthy and MS subjects was carried out in the IBRC in 2018. The healthy individuals were selected from people without any signs and symptoms of autoimmunity or underlying diseases. They should not take any immunosuppressive drugs and with no history of MS patients in their family. The MS patients were selected from patients diagnosed with RRMS-type based on 2017 McDonald MS diagnostic criteria. All patients were under treatment and routine care.

Polymerase Chain Reaction (PCR) and genotyping

The extraction of peripheral blood-DNA was performed according to the protocol of the IBRC kit (Iran). The IBRC kit is a DNA-extraction kit produced in the Iranian Biological Resource Center. To ensure the purity of the DNA samples and determine their optimal concentration to produce suitable PCR products, the purified DNA samples were qualitatively and quantitatively analyzed by electrophoresis with agarose gel as well as by the NanoDrop absorption. Until the analysis, the DNA samples were stored at -20°C. Forward and reverse primers for the *ANKRD55* gene were designed using PerlPrimer (v. 1.1.21) and Gene Runner (v. 6.5.50). Table 1 presents the properties of the primers and the specific product size. After completing PCR and checking the quality of specific products, to confirm the mutation on the *ANKRD55* gene, genotyping/sequencing analysis was performed on PCR products by the Sanger sequencing system. The results were analyzed for the upstream analysis of the *ANKRD55* gene using the finch TV (v. 1.4.0) software and nucleotide BLAST.

Statistical analysis

The patients' and healthy subjects' demographic characteristics and alleles were compared using the Chi-square test. The genotypic frequencies in both controls and patients were adjusted to the proportions expected by the HWE. Multiple logistic regression analysis was conducted to confirm the association between the risk of MS and the *ANKRD55* rs6859219 SNP, with the dependent variable of MS and the confounding variables of age, gender, smoking status, drug allergy, exposure to chemicals, and *ANKRD55* rs6859219 SNP. Statistical analysis of the data was conducted using SPSS for Windows, version 24 (IBM Corp., Armonk, NY, USA).

Results

ANKRD55 rs6859219 genotype analysis

Forty RRMS patients as cases (12 males and 28 females) and 40 healthy participants as controls (15 males and 25 females) were recruited in the current study.

Table 2. Genotype and alleles frequencies of the rs6859219 polymorphism

Single Nucleotide Polymorphisms	Genotype/Allele	No. (%)		P
		Case (n=40)	Control (n=40)	
rs6859219	GG	25 (62.5)	34 (85)	0.04
	GT	13 (32.5)	4 (10)	
	TT	2 (5)	2 (5)	
Allele	G	63 (79)	72 (90)	0.06
	T	17 (21)	8 (10)	

Caspian Journal of Neurological Sciences

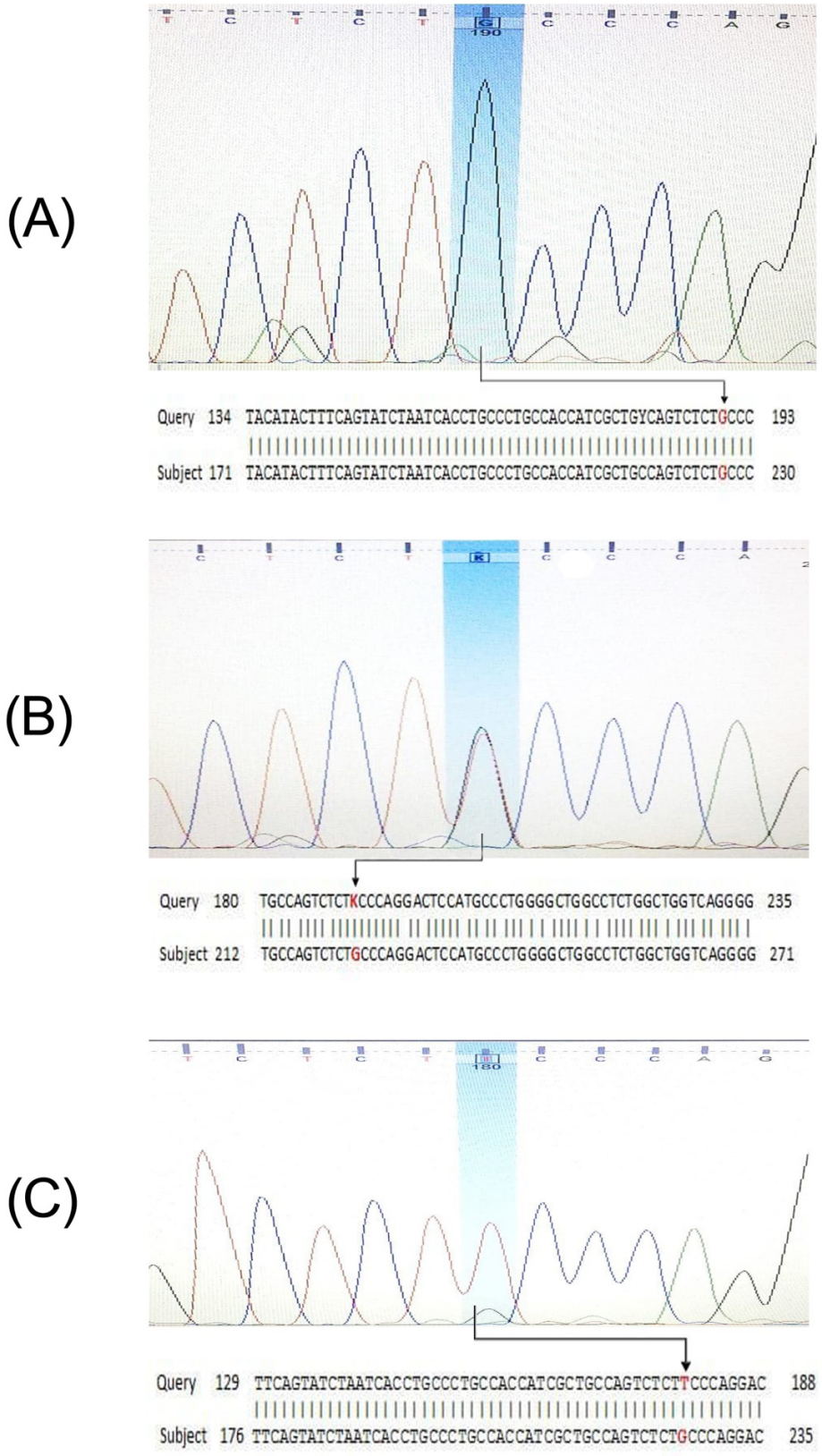

Figure 1. A representative chromatogram related to *ANKRD55* gene polymorphism

A. Gene sequencing of GG genotype; B. Gene sequencing of GT genotype; C. Gene sequencing of TT genotype.

Their Mean±SD ages in the case and control groups were 33±6.16 and 34±13.4 years, respectively, and their minimum and maximum age ranges were 8 and 71 years old. Furthermore, There were no statistically significant differences between the MS cases and healthy controls according to age (P=0.61).

Table 2 presents the comparison of genotype and allele frequencies of the *ANKRD55* rs6859219 polymorphism for MS case and control groups. According to Table 2, the GT genotype was higher in the case group than the other genotypes compared to the control group. In the single-variable association analysis between genotypes and MS, the Chi-square test showed a significant association between MS and the rs6859219 polymorphism (6.138, P=0.04). Furthermore, allele G had a higher frequency in both case and control groups (G allele [in cases]=79%, G allele [in controls]=90%) (Table 2). Figure 1 demonstrates the chromatograms related to *ANKRD55* gene sequencing.

Association between *ANKRD55* rs6859219 polymorphism and the Risk of MS

Table 3 indicates the effect of the *ANKRD55* rs6859219 polymorphism on the MS risk using the additive genetic model. The additive genetic effect was performed to reveal the significant role of rs6859219 genotype in increasing the risk of MS via simple logistic regression. After adjusting each confounder to the additive model to observe a correlation between *ANKRD55* rs6859219 polymorphism and MS, our findings indicated that the rs6859219 genotype significantly increased the risk of MS. Using the logistic regression analysis to determine the relationship between other variables such as age, gender, smoking status, drug allergy, and exposure to chemicals, we concluded that in the presence of other variables, the association between genotype and MS was significant (P<0.05). Moreover, a significant relationship was found between factors such as gender, age, smoking status, drug allergy, and exposure to chemicals and the disease (P=0.01, P=0.01, P=0.02, P=0.01, and P=0.02 respectively) (Table 3).

According to Table 4, the effects of the rs6859219 polymorphism were investigated in three genetic models (dominant, recessive, and overdominant). Multiple logistic regression analysis was performed to confirm the association between the risk of MS and *ANKRD55* rs6859219 polymorphism, with the dependent variable as MS and the confounding variables as age, gender, the status of smoking, drug allergy, chemicals exposure, and *ANKRD55* rs6859219 genotypes. Results of all crude models, additive, recessive, dominant, and overdominant, were significantly different in participants with MS compared to the control group (P=0.01, 1, P=0.02,

Table 3. Genetic association with adjustment for each confounding variable in the MS cases and healthy controls

Variables		Groups/No. (%)		Crude		Adjusted*	
		Control	Case	P	OR (95% CI)	P	OR (95% CI)
Genotypes	GG	34 (85)	25 (62.5)	0.04	-	-	-
	GT	4 (10)	13 (32.5)				
	TT	2 (5)	2 (5)				
Gender	Male	15 (37.5)	12 (30)	0.47	1.4 (0.5-3.5)	0.01	4.3 (1.27-15.11)
	Female	25 (62.5)	28 (70)			0.7	1.4 (0.18-11.02)
Age (y)	<35	20 (50)	25 (62.5)	0.26	0.6 (0.2-1.4)	0.01	4.4 (1.27-15.28)
	>35	20 (50)	15 (37.5)			0.8	1.2 (0.15-9.5)
Smoking status	No	26 (65)	20 (50)	0.17	1.8 (0.7-4.5)	0.02	4 (1.1-14.09)
	Yes	14 (35)	20 (50)			0.9	1.1 (0.14-9.01)
Drug allergy	No	39 (97.5)	38 (95)	0.50	2.05 (0.17-23.5)	0.01	4.6 (1.3-16.12)
	Yes	1 (2.5)	2 (5)			0.7	1.4 (0.18-10.93)
Exposure to chemicals	No	37 (92.5)	35 (87.5)	0.45	1.7 (0.39-7.92)	0.02	4.2 (1.22-14.83)
	Yes	3 (7.5)	5 (12.5)			0.8	1.1 (0.13-10.32)

*Adjustment was made for age, gender, smoking status, drug allergy, and exposure to chemicals.

P<0.05 were considered statistically significant.

Table 4. The effects of the rs6859219 polymorphism in the risk of MS using three genetic models

Genetic Models	Unadjusted		Adjusted*	
	OR (95%CI)	P	OR (95%CI)	P
Additive				
GG (Ref)	-	-	-	-
GT	4.42 (1.28-15.18)	0.01	3.8 (1.06-13.69)	0.04
TT	1.36 (0.17-10.23)	0.76	0.99 (0.10-9.31)	0.99
Recessive (GG+GT) vs TT	1 (0.13-7.47)	1	0.6 (0.06-5.52)	0.65
Dominant (GT+TT) vs GG	0.29 (0.10-0.86)	0.02	0.33 (0.10-1.06)	0.06
Overdominant (GG+TT) vs GT	4.33 (1.27-14.77)	0.01	3.94 (1.11-14.01)	0.03

*Adjustment was made for age, sex, smoking, drug allergy, and chemicals.

CJNS

P<0.05 were considered statistically significant.

and P=0.01, respectively) (Table 4). The GT genotype significantly increased the risk of MS compared to the TT genotype (OR=4.42, 95%CI: 1.28–15.18; P=0.01). A combination of rs6859219 GT genotype and each confounder such as age, gender, smoking status, drug allergy, and exposure to chemicals carried a higher risk of MS than GG or TT genotypes.

Logistic regression analysis demonstrated a significant association between allele frequencies of the *ANKRD55* rs6859219 polymorphism and the risk of MS in participants with MS in additive and overdominant genetic models except for the recessive model (P<0.05) (Table 4). Our findings indicated a significant correlation between MS and the *ANKRD55* rs6859219 polymorphism in the additive genetic model (unadjusted model [P=0.01, 0.7; GT, TT]) adjusted for age, gender, smoking status, drug allergy, and exposure to chemicals (P=0.04, 0.99; GT, TT), and in the overdominant genetic model (unadjusted model [P=0.01; GG-TT vs GT]) adjusted for age, gender, smoking status, drug allergy and exposure to chemicals (P=0.03; GG-TT vs. GT). However, no significant association was found between the *ANKRD55* rs6859219 polymorphism and the risk of MS with and without adjusting confounders (P>0.05). Surprisingly, in the dominant genetic model, the GT-TT genotype decreased the risk of MS compared to the GG genotype (OR=0.33, 95%CI: 0.10-1.06; P=0.02).

Discussion

In the current study, the effects of the *ANKRD55* gene SNP (rs6859219) on the risk of MS were investigated in Iranian patients. Our results indicated that the level of the allele and genotype distribution of rs6859219 of this gene in patients was higher than in healthy subjects. The HWE test was conducted on the control group showed the goodness of fit of HWE in the rs6859219 polymorphism, demonstrating that the control group was representative and in stable conditions. We found that the GT genotype was increased more in the case group compared to the control one, and allele G had a higher frequency in case and control groups (G allele= 79%, G allele= 90%). After examining the relationship between variables (age, gender, smoking status, drug allergy, and exposure to chemicals) and MS, we found a significant association between the genotype and MS (P<0.05). Furthermore, a significant relationship was found between factors such as gender, age, smoking status, drug allergy, and exposure to chemicals and the disease (P=0.01, P=0.01, P=0.02, P=0.01, and P=0.02, respectively). Additionally, the effects of the rs6859219 polymorphism on three genetic models (dominant, recessive, and overdominant) were examined.

The results of all crude models, additive, recessive, dominant, and overdominant, were significantly different in participants with MS compared to the control group (P=0.01, 1, P=0.02, and P=0.01, respectively). The risk of MS increased significantly by the GT genotype compared to the TT genotype to approximately 4.5 units (OR=4.42, 95%CI: 1.28–15.18; P=0.01). Surprisingly, in the dominant genetic model, GT+TT genotypes were decreased 33% the risk of MS compared to the GG genotype (OR=0.33, 95%CI: 0.10-1.06; P=0.02).

Genetic determinants affecting MS may be employed as a diagnostic or therapeutic factor. Increasing the ex-

pression of the *ANKRD55* gene in nerve cells has been reported in many autoimmune disorders, including rheumatoid arthritis, ulcerative colitis, diabetes type-2, Graves disease, celiac disease, and MS [19, 20]. Furthermore, there is an association between *ANKRD55* gene polymorphism and MS, and it has been recently identified as a novel candidate gene for the risk of MS [9, 10]. Recent research to elucidate the possible role of *ANKRD55* in the inflamed CNS has investigated the *ANKRD55* gene in the mouse model of Encephalomyelitis (EAE) and immune and microglial cells under neuroinflammatory conditions. The researchers concluded that *ANKRD55* was dominant in the nucleus and had preliminary data showing the *ANKRD55* expression in primary rat astrocyte and oligodendrocyte cultures. Moreover, the expression and production of *ANKRD55* protein in response to neuroinflammation were higher in the brain, spinal cord, and spleen of EAE mice than in healthy animals. Their flow cytometry analysis of CNS-infiltrating mononuclear cells showed that CD4+ T cells and monocytes expressed *ANKRD55* in EAE mice and a low percentage of microglia expressed *ANKRD55* and this observation demonstrates that *ANKRD55* is affected by an autoimmune condition of CNS and this requires future studies investigating the role of this gene and protein in MS [20].

The *ANKRD55* gene and its expressed protein are poorly characterized. Ankyrin repeats consist of 33–34 residue motifs conformed in two alpha-helices separated by loops, and they have a considerable function in the protein-protein interconnection. As one of the most abundant motifs in nature, ankyrin repeats are present primarily in eukaryotic proteins with very diverse functions, including transcription factors, cytoskeletal proteins, and cell cycle regulators, among others [22]. Information about the cells and tissues expressing the *ANKRD55* protein and the subcellular location and its function is relatively limited. The human protein atlas has documented the *ANKRD55* expression in many tissues of diverse organ systems, as well as in all myeloid cell lines tested [12, 20, 23]. In few cases, an investigation was also conducted on the rs6859219 polymorphism of the *ANKRD55* gene in patients with MS. Alloza et al. investigated two high-risk genes polymorphism: 1) rs6859219, in promoter *ANKRD55*, and 2) rs12785878, in promoter *DHCR7* that caused autoimmune diseases. They examined this possibility in 2895 patients with MS and 2942 healthy individuals in four separate geographical subgroups of Spain (north, south, east, and center of Spain). They concluded no genetically significant relationship between *DHCR7* and MS. On the contrary, available human transcriptome data demonstrate the *ANKRD55* expression in ovaries, testes, endometrium, and $CD4^+$ T cells, which seems to be likely associated with some autoimmune diseases. However, the specific function and role of *ANKRD55* are still unclear [10].

Another study examining the role of *ANKRD55* in various diseases is the Li et al. investigation. They examined the association of five SNPs in *TNFSF4* (rs2205960, rs844644, and rs844648) and *ANKRD55* (rs6859219 and rs7731626) genes in Interstitial Lung Disease (ILD) and DM-PM in 2297 Chinese individuals [24]. Also, DM-PM is a disease, causing skeletal muscle weakness and cutaneous problems [25]. Although DM/PM often affects the skin and muscles, it can also affect multiple organs, especially the lungs. One of these results demonstrated that the *ANKRD55* polymorphism (rs7731626) was significantly associated with DM-ILD as well as DM/PM-ILD. However, no association was found between DM/PM and SNP rs6859219 of *ANKRD55*, which was related to MS [24].

Our study had some limitations, mainly the small number of harvested samples due to limited access to sufficient patients.

Conclusion

In conclusion, our findings demonstrated that the GT genotype of *ANKRD55* rs6859219 SNP could increase the risk of MS compared to the TT genotype. Finally, to evaluate the significances of our data accurately, we suggest further studies in the larger scale populations.

Ethical Considerations

Compliance with ethical guidelines

All study procedures were done in compliance with the Ethical Guidelines of the Declaration of Helsinki, 2013.

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Authors' contributions

Sepideh Mandegarfard and Arash Pourgholaminejad contributed equally to this study; Conceptualization and

methodology: Seyed Abolhassan Shahzadeh Fazeli; Investigation and formal analysis: Sepideh Mandegarfard; Software and validation: Seyed Abolhassan Shahzadeh Fazeli, Arash Pourgholaminejad, and Sepideh Mandegarfard; Supervision: Seyed Abolhassan Shahzadeh Fazeli, Seyed Mohsen Miresmaeili; Writing the original draft: Sepideh Mandegarfard and Arash Pourgholaminejad; Writing, review, and editing: Arash Pourgholaminejad.

Conflict of interest

The authors declared no conflict of interest.

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