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Association between Genetic Polymorphism of The lncRNA *MIAT* rs1894720 with Ischemic Stroke Risk and lncRNA *MIAT* Expression Levels in The Blood after An Ischemic Stroke: A Case-Control Study

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Abstract

Objective: Genetic aspects can play an essential role in the occurrence and development of ischemic stroke (IS). Rs1894720 polymorphism is one of the eight single nucleotide polymorphisms (SNPs) in the long non-coding RNA (lncRNA) myocardial infarction-associated transcript (*MIAT*) locus. The aim of study is the lncRNA *MIAT* rs1894720 polymorphism decreases IS risk by reducing lncRNA *MIAT* expression.

Materials and Methods: In this case-control study, we studied 232 Iranian patients and 232 controls. The blood samples were collected from patients admitted at different times after stroke symptoms. We enrolled 80, 78, and 74 patients who arrived at the hospital between 0-24, 24-48, and 48-72 hours after the first appearance of symptoms, respectively. DNA genotyping was done by the tetra-primer ARMS-PCR method. Circulating *MIAT* levels were evaluated by real-time polymerase chain reaction (PCR).

Results: The GT genotype of *MIAT* rs1894720 showed a significant association with the risk of IS (OR=3.53, 95% CI=2.13-5.84, P<0.001). *MIAT* expression was higher relative to the control within the first hours after IS. The *MIAT* levels in IS patients with rs1894720 (GT) were significantly lower relative to patients who had the GG and TT genotypes. Linear regression model indicated a significant correlation between *MIAT* expression with atherosclerotic risk factors and types of stroke in IS patients. Receiver operating characteristic (ROC) curve analysis showed that the level of lncRNA *MIAT* after IS could be diagnostic with an area under the curve (AUC) of 0.82. The sensitivity and specificity were 80.17 and 67.24%, respectively (P<0.001).

Conclusion: Our study demonstrated that the *MIAT* rs1894720 polymorphism (GT) might increase the risk of IS in the Iranian population. *MIAT* expression was up-regulated in our IS patients. Hence, it could be a diagnostic biomarker for IS.

Keywords: Biomarkers, Gene Expression, Long Non-Coding RNA

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Introduction

Ischemic stroke (IS) is the leading cause of permanent or enduring disability in adults worldwide. The patient outcome is influenced by demographics, clinical, and genetic variables (1). The heritability of IS has been estimated at 38%, and several studies demonstrated the critical roles of genetic aspects in the different processes of IS pathophysiology (2).

Thus, clarifying a patient's genetic biology in addition to their epigenetics can help the prediction and prognosis of IS. Approximately 2% of the transcribed RNA in the human genome plays a significant role in protein encoding. Long non-coding RNAs (lncRNAs) are a type of RNA that contain more than 200 nucleotides and do not have any role in protein synthesis (3). LncRNAs can act as a primary target for treating various disorders. It is used as a new intervention tool for identifying biomarkers and novel treatments for IS (4).

Myocardial infarction-associated transcript (*MIAT*) is a new locus in 22q12.1 identified for myocardial infarction. In addition, *MIAT* with five exons encodes a spliced lncRNA (5). The physiological roles of this gene have been demonstrated in the differentiation of excitatory neurons in the embryonic brain (6). *MIAT* expression has a significant correlation with the risk and progression of different diseases. *MIAT* up-regulation has been demonstrated in patients with IS (7), myocardial infarction (8), and diabetic cardiomyopathy (9). In

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contrast, previous findings showed that down-regulation of *MIAT* expression was associated with schizophrenia, cataracts (10), and diabetic nephropathy (11).

Genetic polymorphisms can affect lncRNA function and expression levels, and can lead to human disease. Single nucleotide polymorphisms (SNPs) are caused by point mutations that affect only one nucleotide within a genetic sequence that gives rise to different alleles (12).

Several SNPs in the *MIAT* locus have a significant association with myocardial infarction (13), and two SNPs in this locus [rs1894720 SNP with minor allele (T) and rs4274 SNP (genotype AA)] are correlated with schizophrenia (14). One SNP, rs1894720 with the GT and TT genotypes, has a significant association with cataracts (10). The rs1894720 polymorphism is one of the several tag SNPs located in the *MIAT* locus. Rao et al. (14) found that *MIAT* SNP rs1894720 increased the risk of paranoid schizophrenia in the minor T allele in a Chinese population. At the same time, its expression was reduced in patients with schizophrenia. Ghapanchi et al. (15) demonstrated that the rs1894720 polymorphism might be associated with an increased risk of salivary gland tumours in the Iranian population. To the best of our knowledge, no further investigation has been conducted into the relationship between *MIAT* polymorphisms and various diseases in the Iranian population. In 2019, Li et al. (10) reported that the *MIAT* rs1894720 polymorphism might be involved in down-regulating MIAT expression. They can enhance miR-26b and decrease BCL2L2 expression, leading to an increased incidence of age-related cataracts. The results of an experimental study showed a correlation with the SNP rs1894720 *MIAT* and down-regulation of MIAT expression, and an increased risk of age-related hearing loss in dominant, codominant, and recessive genetic models (16).

After IS, cerebral hypoxia acutely alters lncRNA expression profiles so that, in the first 24 hours after a stroke, approximately 3000 lncRNAs are differentially expressed, and alterations in lncRNA expression from 24 hours to 7 days after IS has been reported (17). Zhu et al. (7) reported up-regulation of *MIAT* in IS patients and its correlation with National Institutes of Health Stroke Scale (NIHSS) scores and modified Rankin Scale (mRS).

The receiver operating characteristic (ROC) curve is a probability curve used in binary classification to assess a model's ability to distinguish between positive and negative classes at different threshold levels. The area under the curve (AUC) represents the degree of separability between the classes. The potential marker of *MIAT* expression for IS diagnosis was indicated by ROC curves with 0.84 AUC. Also, *MIAT* expression led to apoptosis of neural cells in IS rats (18), and it has been shown to play a vital role in the development of microvascular dysfunction (9) and up-regulation of proinflammatory cytokines in diabetes mellitus (19).

Based on the findings from these studies, we selected rs1894720 across the whole *MIAT* locus with the hypothesis that the suppressive effect of this SNP with GT or/and TT genotypes on *MIAT* expression could have a protective impact on IS. In recent years there has been a growing demand for identifying disease-associated SNPs and lncRNAs. This study aimed to explore the relationship between the rs1894720 SNP and lncRNA *MIAT* expression and risk of IS in Iranian patients. We also compared circulating MIAT levels in IS patients at different times (0-24, 24-48, and 48-72 hours) after stroke onset, different types of strokes, and various genotypes. The association between *MIAT* expression with clinical characteristics and genotype as well as its potential as a diagnostic marker for IS was also assessed.

Materials and Methods

Study subjects

This was a case-control study of 232 patients and 232 controls conducted from August 2018 to august 2019 at Namazi Hospital, Shiraz, Iran. Recent, initial diagnosis of IS with symptom onset within 24 hours comprised the inclusion criterion. Board certified neurologists obtained the patients' histories, and conducted examinations and assessments to confirm the IS diagnosis. Patient histories involved a thorough interview to gather information about past medical conditions, medications, and family history of neurological disorders. Examinations included a physical exam to assess neurological function, such as muscle strength, reflexes, coordination, and sensory perception. All patients underwent either a brain computed tomography (CT), magnetic resonance imaging (MRI), or both to distinguish the infarction area and exclude the possibility of cerebral haemorrhaging. IS was defined according to the World Health Organization criteria (20). Patients with head trauma, subarachnoid, or intracerebral haemorrhaging were excluded from this study. Patients with vasculitis, arterial dissection, fibromuscular dysplasia, transient ischemic attack, Moyamoya disease, sickle cell disease, different malignancies, or severe inflammation were also excluded.

The control group consisted of a representative sample of the Shiraz population, randomly selected from neighbours who resided closest to the cases and who matched the patients in terms of gender and age. All subjects in this study ranged from 32-90 years of age. Individuals with specific illnesses, brain disorders, or previous strokes were excluded from the control group.

We identified hypertension (Htn) and diabetes mellitus according to defined criteria (21). The stroke severity

was evaluated by NIHSS score at admission, such that increased severity had higher scores (22). The functional outcomes were assessed according to the mRS score at three and six months after admission (blinded to *MIAT* levels). The mRS is a single-item global outcome scale used to assess the functional independence of patients after stroke. Rather than evaluating the observed performance of a specific task, the mRS categorises the patient's level of autonomy based on their ability to perform activities they were able to do before the stroke. The mRS defines seven grades of disability, ranging from 0 to 6. Grade 0 indicates no symptoms, while grade 6 indicates death (23). In our study, we considered an mRS score of 3-6 as an unfavourable functional outcome and the mRS score of 0-2 as a favourable outcome six months after the stroke. IS patients were categorised according to TOAST classification: cardioembolism (CE), small-vessel disease (SVD), large artery atherosclerosis (LAA), other determined aetiology (OD), and undetermined aetiology (UD) (24).

The Islamic Azad University at Kazeroon, Iran Ethics Committee (IR.IAU.KAU.REC.1398.037) approved this study. All participants completed an informed consent (or their proxy respondents) prior to study enrolment. Blood samples were obtained from 232 study patients admitted at three different time points [0-24 (n=80), 24-48 (n=78), and 48-72 (n=74) hours] after onset of initial stroke symptoms.

Rs1894720 single nucleotide polymorphism genotyping and measurement of the *MIAT* lncRNA levels

We used the Favorgen Kit (Taiwan) for DNA extraction. Additionally, the Tetra-ARMS PCR method was employed because it is a fast, inexpensive, and accurate method to assess SNP (25). Primer Express software v.3.0 (Applied Biosystems, Foster City, CA, USA) and the Primer1 program (LAMP web server at: http://primer1.soton.ac.uk/primer-1.html) was applied to design and analyse the oligonucleotide primers. The annealing temperature was 65°C for 30 seconds. The following primers were used to detect the rs1894720 polymorphism in the lncRNA *MIAT*.

Forward outer (FO):
5′TTGGAGAAACTAGAGGCCTGACAGTCG3′

Reverse outer (RO):
5′TAGGTTAATCACACCATGCAACACTGCC3′

Forward inner (FI):
5′CAATAAATAGGGAAGCAACATGCTTTTAGG3′

Reverse inner (RI):
5′AATCAACCCTAACCACATGGACCCCGA3′

SNP was located at chromosome 22:26671261. The size of products included the outer primers (421 bp), G allele (189 bp), and T allele (288 bp). The outer primers are not allele specific and are used to amplify the region that comprises the SNP.

RNA was extracted from whole blood samples with an RNA extraction kit (Favorgen, Taiwan) according to the manufacturer's instructions. RNA samples that had A260/A230 and A260/A280 ratios greater than 1.7 were chosen for cDNA synthesis. The quantitative real-time PCR test was used to measure the level of lncRNA *MIAT*. A Quantstudio 3 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) was used with the following primers:

MIAT-
F: 5′- TCCCATTCCCGGAAGCTAGA -3′
R: 5′- GAGGCATGAAATCACCCCCA -3′

We used primers from previous studies for the TATA box-binding protein (*TBP*) (26). Cycle threshold (Ct) values were used to present variations in expression levels. The Ct difference between *TBP* and *MIAT* was shown by ΔCt. Relative *MIAT* expression level was defined using $2^{-\Delta Ct}$ for each subject.

Statistical analysis

The chi-square test was used to assess Hardy-Weinberg equilibrium (HWE) in the control group. The independent two-sample t-test and a chi-square test were used to evaluate the differences between numeric variables and categorical data, respectively. The association between the risk of IS and *MIAT* polymorphisms was shown by the odds ratio (OR) and corresponding 95% confidence interval (CI). Logistic regression analyses were conducted to evaluate *MIAT* expression levels and clinical parameters between the cases and controls. Analysis of variance (ANOVA) was used to compare the *MIAT* expression levels between different time points after stroke and various types of IS. *MIAT* expression levels are shown as mean ± SE. Relationships between MIAT levels and clinical parameters and genotypes were tested using subgroup analysis and linear regression. We used the Spearman correlation to assess for correlations between stroke severity and *MIAT* levels. Diagnostic and prognostic potential was assessed by ROC curve analysis with AUC serving as a measure of performance. Analysis was performed with SPSS software version 19.0 (IBM SPSS Inc., USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ indicated statistical significance.

Results

Participants' demographic and clinical parameters

The case group included 232 IS patients comprised of 94 females (40.5%) and 138 males (59.5%) who were 32 to 90 years of age (65.90 ± 14.44 years). Table 1 lists the demographic and clinical characteristics of all subjects. The laboratory results showed a significant difference between the cases and controls in terms of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) levels.

Table 1: Demographic and clinical characteristics of the studyparticipants

Characteristics	IS patients (n=232)	Controls (n=232)	P value
Male	138 (59.48)	138 (59.48)	0.99[a]
Female	94 (40.51)	94 (40.51)	
Age (Y)	65.90 ± 14.44	65.90 ± 14.44	0.99[b]
BMI (kg/m^2)	25.99 ± 4.37	26.24 ± 4.48	0.68[b]
Htn			<0.001[a]
Yes	134 (57.75)	62 (26.72)	
No	98(42.24)	170 (73.27)	
Diabetes			0.002[a]
Yes	72 (31.03)	32 (13.79)	
No	160 (68.96)	200 (86.20)	
Smoking			<0.001[a]
Yes	70 (30.17)	16 (6.89)	
No	162 (69.82)	216 (93.10)	
Drinking			0.003[a]
Yes	34 (14.65)	6 (2.58)	
No	198 (85.34)	224 (96.55)	
HLP			0.003[a]
Yes	82 (35.35)	42(18.10)	
No	150 (64.65)	190(81.89)	
TG (mg/dL)	138.72 ± 61.29	135.68 ± 64.03	0.71[b]
TC (mg/dL)	149.37 ± 67.28	153.71 ± 35.206	0.51[b]
LDL (mg/dL)	99.61 ± 37.71	84.99 ± 30.41	0.001[b]
HDL (mg/dL)	49.37 ± 28.8	43.60 ± 10.9	0.04[b]
TOAST classification			
LAA	68 (29.31)		
SVD	52 (22.41)		
CE	54 (23.27)		
UD	58 (25.00)		
NIHSS (admission)			
≤6	128 (55.17)		
≥7	104 (44.83)		
mRS (3 months)			
0-2	68 (29.31)		
3-6	164 (70.68)		

Data are shown as mean ± standard deviation (SD) or n (%). Htn was defined as systolic blood pressure ≥140 mmHg and/ or diastolic blood pressure ≥90 mmHg or the use of antihypertensive agents. Diabetes mellitus was diagnosed based on the following criteria: two fasting glucose levels >126 mg/dl (7.0 mmol/L) and two hours post-load glucose >200 mg/dl (11.1 mmol/L) or treatment with hypoglycaemic drugs. a; Chi-square test, b; Independent two-sample t test, IS; Ischemic stroke, BMI; Body mass index, TG; Triglycerides, TC; Total cholesterol, LDL; Low-density lipoprotein, HDL; High-density lipoprotein, NIHSS; National Institutes of Health Stroke Scale, mRS; Modified Rankin Scale, LAA; Large artery atherosclerosis, SVD; Small-vessel disease, CE; Cardioembolism, UD; Undetermined, HLP; Hyperlipidaemia, and Htn; Hypertension.

MIAT rs1894720 polymorphism and the risk of ischemic stroke

PCR product size for lncRNA *MIAT* rs1894720 polymorphism was 189 bp for the G allele, 288 bp for the T allele, and 421 bp for the internal control on 2% agarose gel (Fig.1). Allele frequencies and genotypes of the *MIAT* rs1894720 polymorphism were evaluated in both cases and controls (Table 2). We did not find any significant differences in allele frequency of the *MIAT* gene between the case and control groups (P=0.69). In addition, the patients with IS were genotyped as GG (n=28), GT (n=188), and TT (n=16). The GT genotype was associated with a 3.53-fold increase in IS risk in a codominant model (OR=3.53, 95% CI=2.13-5.84, P<0.001). In the dominant model, the GT+TT genotypes were associated with a 2.60-fold higher risk of IS (OR=2.60, 95% CI=1.59–4.25, P<0.001). Also, in the over-dominant model, the GT genotypes were related to a 4.27-fold higher risk of IS (OR=4.27, 95% CI=2.82-6.48, P<0.001). The *MIAT* rs1894720 polymorphism was associated with a 0.24-fold decrease in IS risk in the recessive model (TT vs. GG+GT genotypes) (Table 2). No significant deviations from the HWE were observed in the rs1894720 polymorphism in the control group (P=0.05).

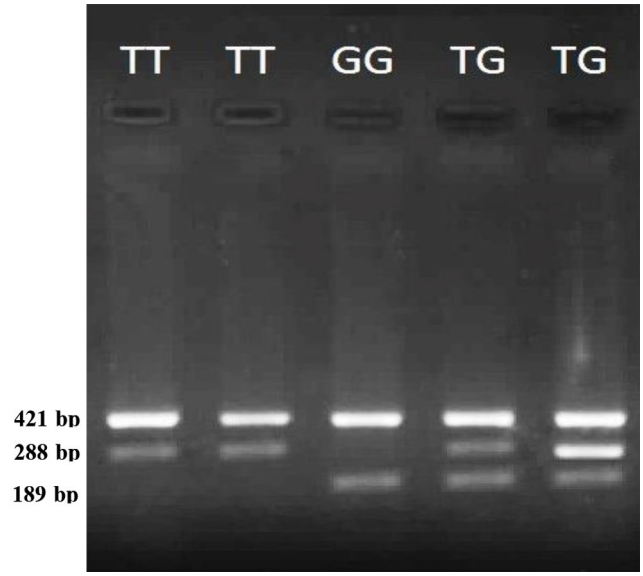

Fig.1: Tetra-primer ARMS-PCR for the detection of lncRNA *MIAT* rs1894720 polymorphism. The product sizes were 189 bp for the G allele, 288 bp for the T allele, and 421 bp for the internal control. lncRNA; Long non-coding RNA.

Alterations in the level of *MIAT* lncRNA in ischemic stroke patients relative to controls

Figure 2A shows a significantly high expression level of *MIAT* in all IS patients relative to controls

(3.08 ± 0.26 vs. 1.05 ± 0.09, P<0.001). Figure 2B shows significant up-regulation of *MIAT* expression in IS patients compared to the controls at 0–24 hours (2.98 ± 0.43 vs. 1.01 ± 0.16), 24-48 hours (3.61 ± 0.53 vs. 1.1 ± 0.19), and 48-72 hours (2.63 ± 0.38 vs. 1.04 ± 0.13) (all P=0.001). *MIAT* up-regulation remained elevated until 72 hours after stroke onset. There was no significant difference between *MIAT* expression at the three time points [F (2, 113)=1.137, P=0.3246]. Further analysis revealed that *MIAT* expression was significantly (P<0.05) lower in IS patients who had the rs1894720 (GT) polymorphism relative to the GG, TT genotypes, 2.82 ± 0.25 vs. 4.18 ± 0.87, respectively (Fig.2C). We found significant *MIAT* up-regulation in LAA (P<0.001), SVD (P<0.01), CE (P<0.01), and UD (P<0.05) IS relative to the controls, while there were no significant differences between *MIAT* levels in the four subgroups [F (3, 112)=1.932, P=0.12] (Fig.2D). Logistic regression analysis demonstrated a positive association between *MIAT* expression and the risk of IS. The increase in *MIAT* score remained significant even after adjusting for related variables of body mass index (BMI), Htn, diabetes, hyperlipidaemia (HLP), alcoholism, and smoking (P<0.001; adjusted OR=1.599; 95% CI=1.21-1.99).

Association of *MIAT* expression with clinical parameters, genotype, and type of stroke

We used subgroup analyses to assess the correlation between *MIAT* expression with clinical parameters and genotype (Table 3). *MIAT* up-regulation was found in the Htn and diabetes subgroups (P<0.01), as well as in the HLP and GT genotype subgroups (P<0.05). However, no significant differences were found regarding sex, age, BMI, NIHSS, mRS, smoking, and alcoholism. In addition, linear regression analysis was employed to identify the association between lncRNA *MIAT* levels and clinical parameters, as well as stroke types, in the cohort of 232 patients (Table 4). There was a significant positive relationship between *MIAT* expression with diabetes (P=0.01), Htn, (P=0.005), drinking (P=0.04), and LAA (P=0.01) in IS patients.

Table 2: Distribution of genotypes, allele frequency of the rs1894720 MIAT gene polymorphism and OR with 95% CI in cases and healthy controls

Inheritance Model	rs1894720 Polymorphism	Cases (%)	Controls (%)	OR (95% CI)	P value
Codominant[a]	GG	28 (12.06)	61 (26.29)	1	
	GT	188 (81.03)	116 (50)	3.53 (2.13-5.84)	<0.001
	TT	16 (6.89)	55 (23.71)	0.63 (0.31-1.29)	0.20
Dominant[b]	GG	28 (12.06)	61 (26.29)	1	
	GT+TT	204 (87.93)	171 (73.71)	2.60 (1.59-4.25)	<0.001
Recessive[c]	GG+GT	216 (93.10)	177 (76.29)	1	
	TT	16 (6.89)	55 (23.71)	0.24 (0.13-0.43)	<0.001
Over-dominant[d]	GG+TT	44 (18.96)	116 (50)	1	
	GT	188 (81.03)	116 (50)	4.27 (2.82-6.48)	<0.001
	G	244 (52.58)	238 (51.29)	1	
	T	220 (47.41)	226 (48.70)	0.94 (0.73-1.22)	0.69

The relationship of each SNP with case/control status was determined by employing unconditional logistic regression analyses. The association between the risk of IS and *MIAT* polymorphisms is shown by OR and the corresponding 95% CI. IS; Ischemic stroke, OR; Odds ratio, CI; Confidence interval, SNP; Single nucleotide polymorphism, [a]; Codominant: Major allele homozygotes vs. heterozygotes, [b]; Dominant: Major allele homozygotes vs. heterozygotes+minor allele homozygotes, [c]; Recessive: Major allele homozygotes+heterozygotes vs. minor allele homozygotes, and [d]; Over-dominant: Major allele homozygotes+minor allele homozygotes vs. heterozygotes.

Spearman correlation of lncRNA *MIAT* with National Institutes of Health Stroke Scale in ischemic stroke

The level of *MIAT* expression in patients showed a positive, nonsignificant correlation with NIHSS scores (r=0.03, P=0.61). We found a difference in the correlation of *MIAT* expression between IS patients with rs1894720 (GT) and patients with other genotypes (GG, TT). We found a Pearson's correlation coefficient close to zero in patients with the GT genotype, which indicated no correlation between the variables (r=0.05, P=0.59) and a positive nonsignificant linear weak correlation in patients with the GG, TT genotype (r=0.38, P=0.07). In patients with the GT genotype, decreased *MIAT* expression did not have any significant relationship between *MIAT* level and NIHSS score. Patients with the GG, TT genotype and a higher *MIAT* level had a weak positive correlation between *MIAT* level and NIHSS.

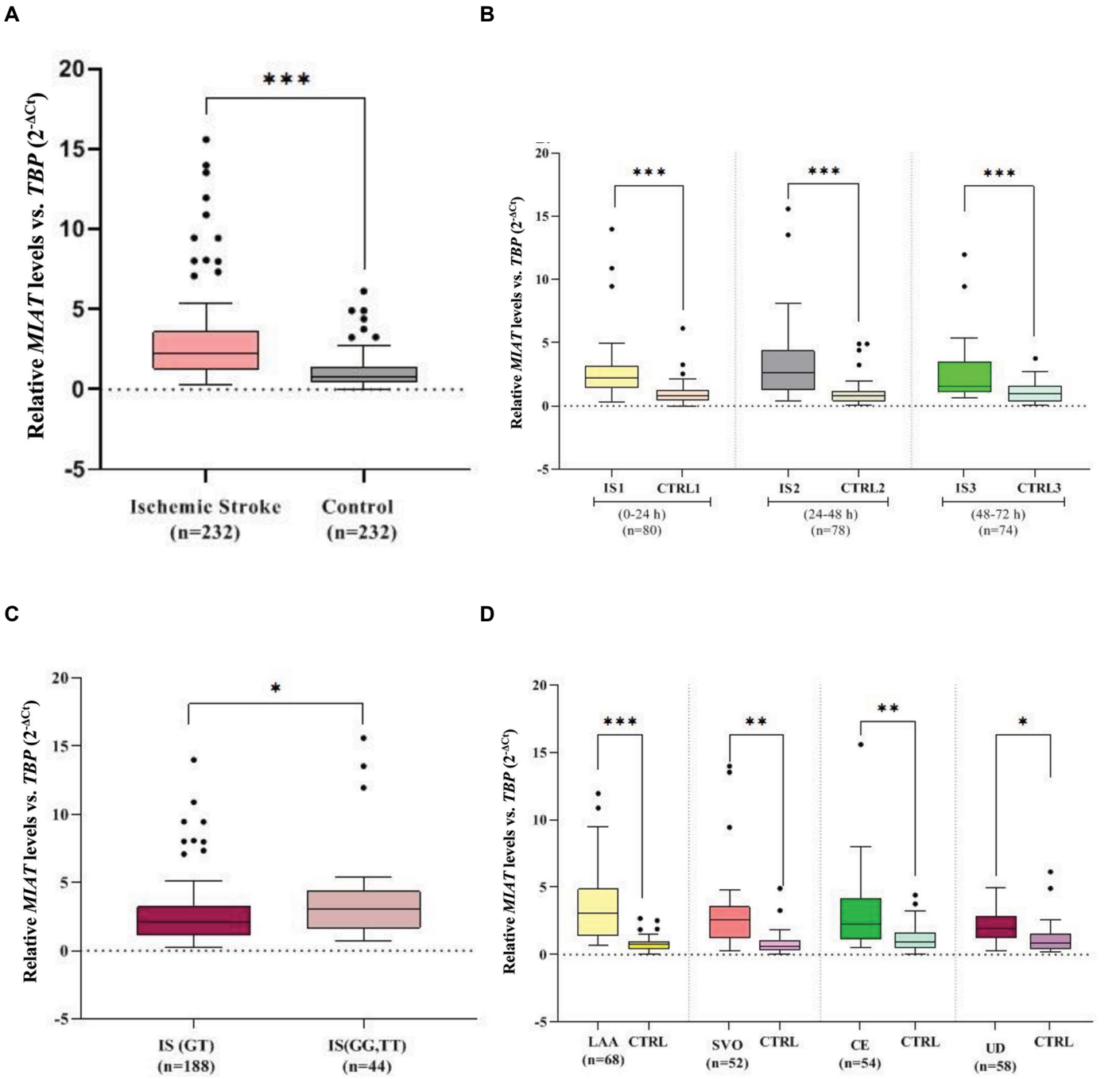

Fig.2: The expression levels of *MIAT* in different subgroups. **A.** Independent Student's t test revealed that in all IS patients, lncRNA *MIAT* levels were significantly higher than in the controls. **B.** At 0-24, 24-48, and 48-72 hours after stroke, the blood levels of lncRNA *MIAT* in IS patients was higher than the age-sex matched controls. There were no significant differences in *MIAT* levels between these three time points. **C**. Comparison of *MIAT* levels between two subgroups of patients with different genotypes GT relative to GG, TT. **D.** *MIAT* levels in the LAA, CE, SVD, and UD groups were significantly higher than in the matched controls. One-Way ANOVA analysis showed no differences among types of strokes. Results are expressed as mean ± SEM. *; P<0.05, **; P<0.01, ***; P<0.001, IS; Ischemic stroke, lncRNA; Long non-coding RNA, LAA; Large artery atherosclerosis, SVD; Small-vessel disease, CE; Cardioembolism, ANOVA; Analysis of variance, CTRL; Control, UD; Undetermined, SVO; Small vessel occlusion, and h; Hour.

Table 3: Association between clinical parameters and genotype with lncRNA *MIAT levels in IS patients*

Characteristics	Number	Mean ± SD	t	P value
Sex			0.161	0.82
Male	138	3.12 ± 2.83		
Female	94	3.03 ± 2.93		
Age (Y)			0.350	0.93
<70	130	2.86 ± 2.43		
≥70	102	3.36 ± 3.35		
BMI (kg/m^2)			0.680	0.49
<24	82	3.23 ± 3.06		
≥24	150	2.95 ± 2.75		
NIHSS			1.513	0.13
≤6	128	2.72 ± 2.16		
≥7	104	3.52 ± 3.51		
mRS (admission)			1.406	0.16
0-2	66	2.94 ± 1.68		
3-6	166	3.31 ± 3.19		
mRS (6 months)			0.492	0.62
0-2	148	3.18 ± 2.98		
3-6	84	2.91 ± 2.66		
mRS (3 months)			0.550	0.58
0-2	122	2.97 ± 2.91		
3-6	110	3.27 ± 2.84		
Smoking			0.233	0.81
Negative	162	3.125 ± 3.11		
Positive	70	2.98 ± 2.22		
Alcoholism			1.85	0.06
Negative	198	2.88 ± 2.53		
Positive	34	4.25 ± 4.21		
Hypertension			2.47	0.008**
Negative	98	2.33 ± 1.69		
Positive	134	3.63 ± 3.38		
HLP			1.88	0.03*
Negative	150	2.41 ± 1.83		
Positive	82	3.45 ± 3.24		
Diabetes			2.74	0.007**
Negative	160	2.02 ± 1.44		
Positive	72	3.56 ± 3.20		
Genotype			2.029	0.04*
GT	188	2.82 ± 2.45		
GG, TT	44	4.18 ± 4.09		

The data were analysed using the Student's t test. P<0.05 indicates statistical significance.IS; Ischemic stroke, lncRNA; Long non-coding RNA, BMI; Body mass index, mRS; Modified Rankin Scale, NIHSS, National Institutes of Health Stroke Scale, and HLP; Hyperlipidaemia.

Table 4: Linear regression analysis for the association between clinical parameters, type of stroke, and genotypes with lncRNA *MIAT* levels in IS patients

Variables	Beta	95% CI		P value
Genotypes	-0.106	-2.081	0.536	0.24
Age (Y)	-0.040	-0.046	0.030	0.68
Sex	0.093	-0.570	1.655	0.33
HLP	0.139	-0.259	1.916	0.13
DM	0.228	0.264	2.544	0.01
Htn	0.254	0.453	2.481	0.005
Smoking	-0.088	-1.726	0.639	0.36
Drinking	0.194	0.060	3.067	0.04
NIHSS	-0.009	-0.086	0.078	0.92
LAA	0.277	0.394	3.083	0.01
SVD	0.176	-0.292	2.704	0.11
CE	0.135	-0.607	2.426	0.23

Linear regression analysis was done. Bold values denote statistical significance at the P<0.05 level. lncRNA; Long non-coding RNA, IS; Ischemic stroke, NIHSS; National Institutes of Health Stroke Scale, Htn; Hypertension, HLP; Hyperlipidaemia, LAA; Large artery atherosclerosis, SVD; Small-vessel disease, CE; Cardioembolism, UD; Undetermined, and DM; Diabetes mellitus.

Diagnostic potential ,prediction of functional outcome, and mortality of *MIAT* expression level in ischemic stroke

The potential diagnostic marker of lncRNA *MIAT* was assessed by ROC curve analysis. The results indicated that *MIAT* is a potential marker with an AUC of 0.82 ± 0.026 (95% CI=0.776-0.881, Fig.S1A, See Supplementary Online Information at www.celljournal.org), with a sensitivity and specificity of 80.17 and 67.24%, respectively (P<0.001). *MIAT* levels showed high diagnostic potential for IS. In our study, patients with an mRS score of 3-6 within six months after stroke were considered to have an unfavourable functional outcome. We did not find a significant predictive prognosis of *MIAT* levels when we analysed the ROC curve for negative outcomes relative to a favourable outcome with an AUC of 0.60 ± 0.05 (95% CI=0.447-0.657, Fig.S1B, See Supplementary Online Information at www.celljournal.org). The sensitivity and specificity were 70.37 and 45.16%, respectively.

At the six month follow-up, 56 cases (24.13%) were deceased. Survivors had lower *MIAT* expression levels than the deceased patients; however, this difference was insignificant. Among other factors, age, BMI, NIHSS score, and mRS score significantly differed between the surviving and deceased patients. *MIAT* expression did not show any significant potential for prediction of six month mortality. The AUC was 0.60 ± 0.06 (95% CI=0.459-0.717, Fig.S1C, See Supplementary Online Information at www.celljournal.org), with a sensitivity and specificity of 90.00 and 35.42%, respectively.

Discussion

This study represents an association of *MIAT* rs1894720 (GT) with a higher risk of IS in Iranian subjects (OR=3.53, 95% CI=2.13-5.84, P<0.001). We observed elevated *MIAT* levels in all of the patients compared to the controls. In contrast, patients with the GT genotype exhibited significantly lower *MIAT* expression levels compared to those with the GG and TT genotypes. The rs1894720 polymorphism is one SNP in the *MIAT* locus, and previous studies have shown an association with *MIAT* down-regulation in the GT and TT genotypes (14, 16). The rs1894720 SNP (GT, TT) in chronic disorders such as schizophrenia and cataracts may be related to a reduction in *MIAT* expression compared to healthy subjects (10, 14). The lower *MIAT* expression in GT patients relative to the GG, TT may be related to the *MIAT* rs1894720 SNP (GT). IS CT an acute neurological disorder, and different factors after cerebral hypoxia acutely and severely alter the expressions of several thousand lncRNAs (17). Cerebral ischemia is an important factor that acutely increases the circulating level of *MIAT* in IS patients (7), while the possible suppressive effect of *MIAT* rs1894720 SNP on *MIAT* expression could reduce *MIAT* levels in IS patients with the GT genotype relative to GG, TT. The suppressive effect of *MIAT* on the expressions of microRNAs-26b and -29b was previously reported (10, 16). *MIAT* expression could be down-regulated due to the GT genotype, and, therefore cause an increase in the expressions of genes that induce neuronal cell death, such as caspase-3, by enhancing miRNA-26b (10). In order to confirm this result, further research needs to be conducted on a larger sample size by analysing plasma microRNAs.

The positive association between *MIAT* expression and cardiovascular diseases [9], as well as atherosclerosis (27), confirms our result about the correlation of lncRNA *MIAT* with LAA stroke. Yan et al. reported the involvement of lncRNA *MIAT* in the pathogenesis of microvascular diseases and angiogenesis (9). lncRNA *MIAT* is highly expressed in atherosclerotic plaque (28). *MIAT*/miR-149-5p/CD47 is a vital macrophage pathway in the growth of necrotic atherosclerotic plaques (29). Therefore, it is possible that the high expression of *MIAT* in atherosclerotic plaques may be down-regulated in subjects with *MIAT* rs1894720 (GT) and this leads to a decrease in the growth of atherosclerotic plaques and the risk of IS. Further investigation in patients with atherosclerosis is needed to confirm this result.

In this study, we demonstrated up-regulated *MIAT* expression after a stroke. Zhu et al. (7), for the first time, showed significant up-regulation in *MIAT* expression levels at 0–24 and 24–48 hours after stroke symptoms. In this study, we evaluated the *MIAT* expression level until 72 hours. The changes in expression levels of different lncRNAs were observed to seven days after a stroke

(17). Yan et al. (9) showed that up-regulation of MIAT was associated with retinal neovascularization, vascular leakage, inflammation, and endothelial dysfunction through the miR-150-5p/VEGF network. Endothelial cell injury is the first step in cardiovascular disease (30). Moreover, Vausort et al. (31) reported a correlation in peripheral *MIAT* expression levels with a marker of myocardial infarction. Another study showed that lncRNA *MIAT*, as a profibrotic factor, regulates cardiac fibrosis and cardiac function in myocardial infarction (8). *MIAT* is localized to specific neurons in the nervous system, including the large cortical neurons and the CA1 region of the hippocampus (32). Neurovascular dysfunction and inflammation can be related to IS pathogenesis (33). Additionally, *MIAT* expression was associated with high-sensitivity C-reactive protein (CRP, r=0.309, P<0.001) as an inflammatory marker in IS patients (7), and neurovascular remodelling in the brain was related to *MIAT* expression (34). These studies indicated that *MIAT* could encourage IS progression.

The up-regulated *MIAT* in our patients showed a non-significant positive correlation with stroke severity, whereas Zhu et al. (7) reported a significant association between lncRNA MIAT levels and NIHSS scores. In our research, subgroup analysis and linear regression did not show a significant correlation between *MIAT* and NIHSS score, whereas we found a Pearson's correlation coefficient close to zero in patients with the GT genotype, which indicated the lack of an association between the variables and a positive non-significant linear correlation in GG, TT patients. *MIAT* expression was lower in our GT patients relative to GG and TT. Thus, according to the possible suppressive effect of this SNP on *MIAT* expression, it is possible that in Iranian patients, we could not see a significant positive correlation between *MIAT* expression and NIHSS score. On the other hand, in the Zhu et al. (7) study, 177 patients were within the first 24 hours and only 12 cases were in the 24-48 hours post-stroke onset. Therefore, the positive association between *MIAT* expression and stroke severity in their study mainly belonged to the expression level of *MIAT* at 0-24 hours, and this time may be an optimal time window for expression of *MIAT*. In our study, there were 78 patients within 24 hours of acute IS; more sample sizes are required and that can show a positive association. It seems that this sample size could show a significant elevation in *MIAT* expression relative to the control. In different types of IS stroke, *MIAT* was equally expressed at a high level, and this also supports the results by Zhu et al. (7). In our study, we detected a significant association between *MIAT* expression with types of strokes (LAA) and atherosclerotic risk factors.

In our study, *MIAT* expression may be a potential biomarker for IS diagnosis (AUC=0.82). *MIAT* is highly expressed in neural cells and the peripheral blood of patients, and it could be considered a biomarker for IS (35) and myocardial infarction (36).

The results of recent studies have demonstrated an involvement of lncRNAs in the pathogenesis and progression of human diseases, as well as their critical regulatory functions. Additionally, genetic variations in lncRNAs have been associated with the risk of various diseases. lncRNAs are potential biomarkers for stroke diagnosis because they are relatively stable biomolecules that can withstand various conditions such as pH, extreme temperatures, and enzymatic breakdown. This stability makes them promising as diagnostic markers that can be detected in multiple body fluids, including blood, cerebrospinal fluid, and urine (37). lncRNAs are expressed differently in stroke patients compared to healthy controls and those with other neurological disorders. This differential expression can be used to identify specific lncRNAs that are associated with stroke and can serve as diagnostic markers (38). lncRNAs can be detected in various body fluids without requiring invasive procedures, such as tissue biopsies, which can be uncomfortable and risky for patients (39). Furthermore, many researchers have found that lncRNAs can be good candidates for molecular biomarkers due to high specificity and sensitivity (40).

This study showed that *MIAT* rs1894720 polymorphism correlated with a higher risk of IS. The present results supported our hypothesis and it estimated, for the first time, an association between SNPs in *MIAT* and IS risk. Additional studies in larger populations are needed to confirm these results.

Genetic association research is becoming increasingly important to identify the genetic underpinnings of complex disorders. Advances in genomics have made it possible to stratify patients according to molecular features thought to play important roles in pathogenesis. Therefore, the diagnosis and treatment of a disease may depend on an individual's gene expression pattern. Personalized medicine is particularly important in the field of IS for improving diagnostic and treatment strategies.

This study has certain limitations. More accurate results can be detected with larger sample sizes. In addition, there are other polymorphisms in lncRNA *MIAT* that need to be investigated. We assessed *MIAT* expression without performing a full transcriptome analysis. lncRNA *MIAT* expression levels were detected in whole blood samples, but not in serum, PBMCs or plasma. Therefore, the origin of lncRNA is still unknown. In addition, we did not evaluate the association of inflammatory biomarkers such as CRP, IL-6, and TNF-α with lncRNA *MIAT*.

Conclusion

The use of blood-based biomarkers is a valuable way to evaluate the risk and prognosis of IS. Our study demonstrated that rs1894720 polymorphism (GT) may increase the risk of IS. At different time points, higher levels of *MIAT* expression were found in IS patients compared with controls. Therefore, this study indicates

that the presence of lncRNA *MIAT* in whole blood could be a diagnostic biomarker for IS. Further investigation of the association between this polymorphism and IS in the larger Iranian population is required to validate these findings.

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Authors' Contributions

T.A.; Performed all of the experiments, data and statistical analysis, and interpreted the data. M.J.M., A.B.-H.; Contributed to the study conception and design, supervised the research and assisted with the study plan. M.B., A.S.; Advised the research and assisted with the research progression. All authors read and approved the final manuscript.

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