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ORIGINAL ARTICLE

Association of MIR34A Gene Polymorphism rs369892834 with Ischemic and Hemorrhagic stroke risk

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ABSTRACT

Stroke is a neurological disorder caused by changes in blood circulation to the brain in the form of ischemic (lack of blood supply) or hemorrhagic (intracerebral bleeding). It is the third cause of death and first cause of permanent disability in the world. This study aimed to investigate the association between rs369892834 polymorphisms in MIR34A with stroke risk. 100 individuals with Persian origin from Northeast of Iran were genotyped for selected polymorphism using ARMS-PCR for determination of allele frequency. The experiment followed with 203 patients with stroke (137 ischemic, 66 hemorrhagic) and 213 controls. Common risk factors for stroke were matched by p-value> 0.05. The genotype frequencies for TT, TC and CC are 52.2%, 30.6%, 17.2% in all patients and 53.5%, 30%, 16.4% in all controls respectively. P-value =0.478 showed no significant association between genetic variants and stroke, but further study in the subgroup of men with ischemic stroke revealed significant association with TT genotype versus TC + CC (P-value = 0.034 OR: 0.485 CI 95%: 0.247-0.951). The results of our study also were significant for CC vs. TT + TC (P-value = 0.032 OR: 0.428 CI 95%: 0.195-0.939). These indicate the C allele increased risk of ischemic stroke in men about 2.042 (P = 0.004). our results showed that there is no significant association between rs369892834 and susceptibility to any types of stroke, but C allele can increase the risk of ischemic stroke in men.

***Keyword**: MIR34A, rs369892834, Stroke, ARMS-PCR*

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INTRODUCTION

Stroke is a neurological disorder caused by disruption in blood circulation to the brain, which can occur in form of lack of blood supply to the cells (ischemic) or caused by bleeding into brain tissue when a blood vessel bursts (hemorrhagic)[1]. approximately 80% of strokes are ischemic and 20% are hemorrhagic[2]. Globally, stroke is the second leading cause of death, it affects 15 million people worldwide and causes 6.7 million death and 5 million permanent disability annually[3, 4]. 50% of stroke deaths occur in Asian countries[5]. It was shown that the incidence of stroke in the North-East of Iran is significantly higher than the incidence in Western countries and also the age of incidence in this area is approximately one decade below the world average[6]. Regarding stroke as a complex disorder, a combination of genetic and environmental factors together may lead to this disease[7, 8]. Lindgren has reviewed efforts

uncovering genetic background of stroke[9]; however more investigations are needed in order to achieve more comprehensive understanding of stroke genetics.
MicroRNAs (miRNAs) are small non-coding RNA molecules with 19-22 base pare long that can have an enormous regulatory role on gene expression by binding to 3'UTR of mRNAs[10]. miRNAs gene are initially transcribed into large pri-microRNA, and then undergo a two-step processing to generate mature miRNAs[11, 12]. Each mature miRNA can regulate a spectrum of targets and it's estimated that expression of more than one-third of human mRNAs are controlled by microRNAs[13]. The seed sequence in mature miRNAs (nucleotide 2-8) has a significant role in miRNA function[12]. Studies have shown contribution of miRNAs in different molecular procedures including molecular etiology and pathology of stroke[14].
Single nucleotide polymorphisms (SNPs) are a common form of variation in human genome, regarded as a reason for personal differences in many features such as susceptibility to disease[15, 16]. Existence of SNP in miRNA gene (miR-SNP) may have effects on biogenesis (if variation occurs on processing sites) and function (if variation occurs within mature miRNA) of miRNAs[17, 18]. Identification of these polymorphisms in populations, helps us in recognition of genetic markers involved in complex diseases and eventually should contribute to dealing with them [12].
MIR34 gene family gives rise to 3 mature isoforms. MIR34A is located in 1p36.22 and the two other isoforms (mir-34b and mir-34c) in chromosome 11[19]. Different investigations on MIR34A function have suggested that this miRNA can have a role in stroke involved procedures[14]. This miRNA seems to play a role in generation of atherosclerotic plaques and vascular diseases[20, 21]. It also can be an actor in stroke pathogenesis since this microRNA is an actor in p53 network and can affect apoptosis [22].
This study aimed to find out the frequency of polymorphisms rs369892834, within mature MIR34A seed side, in Persian population of Northeast of Iran and also to investigate if there is any association between this polymorphism and stroke disease in this area.

MATERIALS AND METHODS

PILOT STUDY

After determining MIR34A as an indicator in stroke via literature review, NCBI Gene database was explored to find out if there is any SNP within the gene or not(www.ncbi.nlm .nih.gov/gene/407040)(www.ncbi.nlm.nih.gov/gene). Rs369892834 T>C was found in position 26 of MIR34A gene (5^{th} nucleotide in mature miRNA). NCBI SNP database (dbSNP) was explored to discover frequency of this polymorphism (http://www.ncbi.nlm.nih.gov/projects/SNP). No frequency data was registered for this variation in dbSNP. To determine power of study, information about frequency is essentials. There for 100 individual with Persian origin from Northeast of Iran was selected and genotyped for mentioned SNP in order to find out allele frequency in our population.

STUDY POPULATION:

After the pilot study the study group was chosen to be comprised of 203 consecutive patients between 15-70 years of age with stroke (137 ischemic stroke, 66 hemorrhagic stroke) who were recruited from Neurology Department, Ghaem Hospital (Mashhad, Iran) between October 2011 and October 2013. A total of 213 unselected, consecutive control subjects with no clinical evidence for any type of stroke were frequency matched for sex, age, lipid profile, Hypertension, diabetes, ethnically and geographically with the patients (P-value < 0.05). The characteristics of the stroke patients and controls are summarized in Tables 1&2. Patients whose stroke was confirmed by CT-Scan and MRI were included. The exclusion criteria were the traumatic brain injury, presence brain tumors, cardiovascular diseases, and chronic inflammatory diseases (pneumonia, middle ear infections, systemic infections with fever, dental infection) and Patients with cytomegalovirus infection, viral or fungal infection that can cause hemorrhagic stroke Also Were excluded from the study, anyone whose stroke has occurred as a secondary symptom of the disease (e.g., stroke due to embolism after open-heart surgery and a stroke caused by preeclampsia). The study received the approval of the hospital Ethics Research Committee and all the investigated participants provided informed consent before sampling.

DNA EXTRACTION & GENOTYPING:

Peripheral blood from 203 patients and 213 controls was collected in EDTA CBC tube and genomic DNA was extracted and purified from whole blood lymphocytes 5PRIME kit according to the manufacturer's instructions. Specific primers for detection of SNP by Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) were designed by using PRIMER 3 software (http://primer3.ut.ee). Specificity of designed primers was checked for human genome by the Primer-BLAST tool(http://www.ncbi.nlm.nih.gov/tools/primer-blast) .Finally, OligoAnalyzer software was used to check the absence of Hairpin, homodimeric and heterodimeric in PCR conditions(http://eu.idtdna.

com/calc/analyzer). Specific primers for human beta-globulin gene were designed in a same way to be used as internal control in ARMS-PCR reaction. The sequences of primers are shown in Table 3.
ARMS-PCR was performed initially on 100 individuals selected for pilot study and then on case and control groups. Micro-tube components and thermal protocol of PCR reaction are available in table 4 and 5. PCR products were loaded directly onto 2% agarose gels (containing green viewer), electrophoreses and visualized by photography under UV illumination. The product sizes for rs369892834 were 216 bp for the C&T alleles, while the product size for the internal control was 890 bp.

STATISTICAL ANALYSIS

The differences between the two groups were compared using the Student's t test for continuous variables and the chi-square ($\chi 2$) test for categorical variables. Allele and genotype frequencies between stroke patients and controls were obtained using the chi-square ($\chi 2$) test. Odds ratio (OR) and 95% confidence intervals (CI) were calculated using logistic regression. $P<0.05$ was considered statistically significant. Statistical analyses were performed with SPSS for Windows software package version 21.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Allele frequencies for rs369892834 obtained from a pilot study on 100 Persians of northeastern part of Iran. These frequencies were 64.5% and 35.5% respectively for T and C allele. 203 cases (137 ischemic, 66 hemorrhagic) and 213 controls were genotyped for SNPs loci rs369892834 (MIR34A) by ARMS-PCR assay. common risk factor such as gender, diabetes mellitus , hypertension, age, LDL, HDL, triglycerides and cholesterol were matched between the controls and hemorrhagic patients; also between the controls and ischemic patients with Pvalue≥0.05(Tables 1&2).
Genotype and allele frequencies in both groups of patient and controls were compared separately and no significant difference ($p> 0.05$) was observed between patients and controls (Tables 6&7). This indicates the MIR34A Rs369892834 polymorphism was not associated with ischemic and hemorrhagic stroke. We also investigated the genotype and allele frequencies of this polymorphism in both men and women. The results showed there were significant differences between subgroup of men with ischemic stroke compared to control group (Table 8).
It seems having at least one T allele reduces the risk of men ischemic stroke about %58 (P-value=0.032 OR: 0.428 CI 95%: 0.195-0.939). In contrast, the C allele may be a risk factor for ischemic stroke in men and increases the risk of ischemic stroke (P-value=0.004 OR: 2.041 CI 95%: 1.253-3.325). We did not find any significant association between this polymorphism and the risk of hemorrhagic stroke (Table 7). To determine additional clinical significance, we performed stratified analyses according to age, diabetes mellitus, hypertension and hyperlipidemia. In the stratified analyses, but no significant association were detected.

Table 1. Clinical characteristics of the participants between Ischemic Stroke and control

	Ischemic Stroke (n = 137)	**Control (n = 213)**	**p value**
Age (mean ± SD)	50.45 ± 13.80	50.76 ± 12.08	**0.951**
Male gender, n (%)	57 (41.6 %)	91 (42.7 %)	**0.836**
Hypertension,n (%)	58 (43.3%)	23 (37.7 %)	**0.464**
Diabetes, n (%)	39 (28.5 %)	46 (21.6 %)	**0.143**
Smoking, n (%)	24 (17.5 %)	35 (16.4 %)	**0.791**
HDL (mean ± SD)	42.52 ± 9.32	43.83 ± 9.15	**0.194**
LDL (mean ± SD)	113.69 ± 33.31	113.27 ± 31.86	**0.905**
TG (mean ± SD)	145.59 ± 104.56	145.96 ± 87.33	**0.658**
Chol(mean ± SD)	179.82 ± 44.70	186.58 ± 39.85	**0.141**

Table 2. Clinical characteristics of the participants between Hemorrhagic Stroke and control

	Hemorrhagic Stroke (n = 66)	**Control (n = 213)**	**p value**
Age (mean ± SD)	53-98 ± 11.74	50.76 ± 12.08	**0.057**
Male gender, n (%)	30 (45.50 %)	91 (42.7 %)	**0.696**
Hypertension,n (%)	33 (50.8%)	23 (37.7 %)	**0.140**
Diabetes, n (%)	17 (25.8 %)	46 (21.6 %)	**0.480**
Smoking, n (%)	8 (12.1 %)	35 (16.4 %)	**0.397**
HDL (mean ± SD)	45.59 ± 10.09	43.83 ± 9.15	**0.184**
LDL (mean ± SD)	122.61 ± 38.84	113.27 ± 31.86	**0.050**
TG (mean ± SD)	126.76 ± 62.73	145.96 ± 87.33	**0.181**
Chol(mean ± SD)	191.08 ± 45.88	186.58 ± 39.85	**0.441**

Table 3. Primer sequence

SNP	Primer	Primer abbreviation	sequence	Amplicon size
MIR34A/ rs369892834	Forward for T allele	Ft	5'-TGT GAG TGT TTC TTT GCC A-3'	216bp
	Forward for C allele	Fc	5'-TGT GAG TGT TTC TTT GCC G-3'	
	Reverse	Rsnp	5'-GCC CTG GAG CTC ACT T-3'	
Internal Control	Forward	ICF	5'-CAA TGT ATC ATG CCT CTT TGC ACC-3'	890bp
	Reverse	ICR	5'-GAC TCA AGG CTG AGA GAT GCA GGA-3'	

Table 4. Micro-tube components of ARMS-PCR reaction.

Component (Concentration)	Volume (µL)
Pre-mastermix	7.5
dH_2o	4.1
Fc (10pmol/µL)/ Ft (10pmol/µL)	0.6
Rsnp (10pmol/µL)	0.6
ICF (10pmol/µL)	0.6
ICR (10pmol/µL)	0.6
DNA	1
total	15

Table 5. Thermal protocol of PCR reaction. Touchdown protocol was used. Step 2 to 4 repeated for 14 cycles and step 5 to 7 repeated for 21 cycles.

	Temperature (ºC)	time	Temperature decrease per cycle
1	95	5 minutes	-
2	95	30 seconds	0
3	65-58	20 seconds	0.5
4	72	40 seconds	0
5	95	30 seconds	0
6	58	20 seconds	0
7	72	40 seconds	0
8	72	5 minuets	-

Table 6. Genotype and allele distributions of miRNA-34a for the patients with IS and the control group

	Ischemic	Control	p	OR	95 % CI
Genotype					
TT	68 (49.6 %)	114 (53.5%)	Reference		
TC	45 (32.8%)	64 (30%)	0.506	1.179	0.726-1.915
CC	24 (17.5%)	35 (16.4%)	0.649	1.150	0.631-2.095
Dominant effect					
TT / TC+CC	68/69	114/99	0.478	0.856	0.557-1.315
Recessive effect					
TT+TC / CC	113/24	178/35	0.791	0.926	0.523-1.638
Allele					
T	181	292	Reference		
C	93	134	0.493	1.120	0.811-1.547

Table 7. Genotype and allele distributions of miRNA-34a for the patients with hemorrhagic stroke and the control group

	hemorrhagic	Control	p	OR	95 % CI
Genotype					
TT	38 (57.6 %)	114 (53.5%)	Reference		
TC	17 (25.8 %)	64 (30%)	0.492	0.797	0.417-1.524
CC	11(16.7%)	35 (16.4%)	0.881	0.943	0.436-2.037
Dominant effect					
TT / TC+CC	38/28	114/99	0.563	1.179	0.675-2.058
Recessive effect					
TT+TC / CC	55/11	178/35	0.964	0.983	0.468-2.065
Allele					
T	93	292	Reference		
C	39	134	0.678	0.868	0.597-1.399

Table 8. Genotype and allele distributions of miRNA-34a for the male patients with IS and the male control group

	Ischemic(male)	Control(male)	p	OR	95 % CI
Genotype					
TT	23 (40.4%)	53 (58.2%)	Reference		
TC	16 (28.1%)	23 (25.3%)	0.248	1.603	0.717-3.582
CC	18 (31.6%)	15 (16.5%)	0.019	2.765	1.191-6.418
Dominant effect					
TT / TC+CC	23/34	53/38	0.034	0.458	0.247-0.951
Recessive effect					
TT+TC / CC	39/18	76/15	0.032	0.428	0.195-0.939
Allele					
T	62	129	Reference		
C	52	53	0.004	2.041	1.253-3.325

DISCUSSION

Stroke is one of the most common causes of death after heart disease and cancer, and major cause of long-term disabilities[3, 6]. Multi-factorial nature of the stroke and the role of genetic variations in susceptibility to the disease, indicate that uncovering information about genetic parameters involved in this disease can be helpful in its management [8],

microRNAs can be involved in various biological and pathological pathways through their regulatory roles. SNPs in miRNA genes can have a significant impact on the normal function of the mature or immature miRNAs[12]. This study aimed to evaluate the association between MIR34A rs369892834 polymorphism with stroke risk.

Hsa-MIR34A gene is located on1p36.22 and the polymorphism rs369892834 T>C is within the seed site of this miRNA. So far no association study has been investigated for this polymorphism and no frequency data is reported for it. Mir34a has been shown to be an important microRNA involved in etiology and pathology of atherosclerotic vascular diseases such as stroke [14, 21, 23]. Therefore, the polymorphism within it, seems to act as a genetic marker for this disease. MIR34A contributes to the formation of atherosclerotic plaques by targeting genes involved in cholesterol metabolism and vascular smooth muscle cell [21, 24-26]. Increasing old endothelial cells has been seen In Atherosclerotic plaques and patients with ischemic heart disease, coronary artery disease and hypertension [21]. MIR34A is one of the molecules involved in vascular aging and have a high expression in primary endothelial cells[27]. Overexpression of the MIR34A induces endothelial cells aging and inhibition of proliferation [28, 29]. Recently, SIRT-1(silent information regulator 1) is known as targets of MIR34A and has been proposed as a modulator for vascular endothelial cell homeostasis that plays a key role in angiogenesis [30, 31]. MIR34A downregulate SIRT-1 and induce vascular muscle cell senescence [24]. As we know, aging is an important risk factor in the development of atherosclerotic and coronary disease[32].

Based on information above we hypothesized that the polymorphism within MIR34A can be a genetic marker for ischemic or hemorrhagic stroke. However, our results showed significant association only with men subgroup of ischemic stroke. This result should probably be due to a series of complicated reasons. First of all, this SNP should be regarded in a context with other genetic markers, since stroke is a complex disease and a combination of genetic factors together may result in this phenomenon. This means the result of present study can be valuable considered with other investigations of genetic polymorphisms[33].

Secondly, microRNAs act in a network and it is probable if other regulatory mechanisms be present to adjust the effect of the polymorphism. Especially in this case which the SNP is within the seed site and can have enormous impact on microRNA function; so it is not unlikely if any mechanism be adapted to compensate this effect. On the other hand, it is better in future studies with larger-scale samples in other racial and ethnic populations be conducted to confirm the current findings. Moreover, although the case and control individuals in the current study were carefully selected and matched, there might be some unknown environmental factors that could not be excluded and have influenced the results of the present study. Nevertheless, our findings emphasize the importance of genetic variation in miRNA genes and further research is needed to get into a clear understanding of this miRNA polymorphism association with human diseases.

CONCLUSIONS

All things considered, there was no significant association between rs369892834 polymorphism in MIR34A gene and the risk of ischemic and hemorrhagic stroke. However further analysis revealed considerable association of the C allele with increased risk of ischemic stroke among men in North East Iranian population of Persian origin. In parallel, this study demonstrates that the TT genotype for this polymorphism is associated with decreased risk of ischemic stroke in male individuals of mentioned population.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest in this study.

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