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Pre-microRNAs single nucleotide variants (rs3746444 A > G and rs2910164 C > G) increase the risk of ischemic stroke in the Egyptian population: a case–control study

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Abstract

Background: Ischemic stroke (IS) is the most leading cause of morbidity and mortality worldwide. Micro RNA (miRNA) genetic variants have been identified as a part of IS non-modifiable risk markers. This study aims to identify the possible association of rs2910164 C > G of pre-miRNA-146a and rs3746444 A > G of pre-miRNA-499 with increased IS risk. C-reactive protein (CRP) was studied as one of the mediators of the genetic disturbance in IS. The study included 100 patients with atherosclerotic IS and 100 age and sex matched healthy controls with more than one risk factor for IS. Variants were evaluated by the real-time polymerase chain reaction technique using TaqMan probes. CRP levels were assayed by immunoturbidimetry method on COBAS analyzer.

Results: Regarding rs3746444 A > G, the G allele, and its containing genotypes (GG and GG + AG) were associated with high IS incidence. Increased CRP levels were found to induce IS by GG and GG + AG genotypes, with a cut value of 7.5 mg/ L in differentiation between AA genotype and GG + AG genotypes. Combining the G allele of rs3746444 A > G with either G or C allele of rs2910164 C > G had enhanced the risk. For rs2910164 C > G, the G allele, and the combined GG + GC genotypes were associated with IS risk elevation with no correlation to CRP levels.

Conclusion: The G involving genetic variants of rs3746444 A > G and rs2910164 C > G were associated with an enhanced IS risk. CRP showed higher levels in GG and AG genotypes of rs3746444 with no relation to rs2910164 genotypes.

Keywords: Stroke, MicroRNA, Risk, CRP

Background

Stroke is the world's second leading cause of mortality [1]. Ischemic stroke (IS) is responsible for about 80% of all strokes [2]. Stroke is a progressive health problem in Egypt, demonstrating a prevalence rate of 922/100.000, with 757/100.000 of ischemic type [3]. It is a complicated disease with multiple interacting factors. Hypertension, hyperlipidemia, and diabetes mellitus (DM) are

considered modifiable predisposing factors, while age, sex, race, and genetic modifiers are enrolled as non-modifiable etiological markers of IS [4]. Genome-wide association study (GWAS) has revealed the role of genetic single-nucleotide variants (SNVs) in IS risk. Also, micro RNAs (miRNAs) genes are significantly involved [5].

miRNAs are small, noncoding ribonucleic acids (RNAs) that interact with 3'-untranslated zones (UTR) in messenger RNAs (mRNAs) to regulate their post-transcriptional expression. miRNAs synthesis starts as a long strand; pri-miRNAs are processed into pre-miRNAs then mature miRNAs by Drosha and Dicer enzymes [6].

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SNVs of mature and immature miRNAs forms are well-identified in stroke risk factors, including atherosclerosis, DM, hypertension [7]. miRNAs activation is involved in IS progression by enhancing inflammation, apoptosis, oxidative stress, and ecotoxicity [8, 9].

The rs3746444 A > G of pre-miRNA-499 leads to structural changes of the miR-499 mature form and disrupts its functional regulation of mRNA [10]. The miR-499 controls multiple inflammatory mediators, including interleukin-17 receptor B (IL-17RB), IL-2R, IL-6, IL-23A, IL-2, IL-18R, and C-reactive protein (CRP). CRP is involved in the vascular insult of ischemic stroke through inducing phagocytosis, apoptosis, complement pathway activation, and nitric oxide (NO) level alteration [11, 12].

The rs2910164 C > G of pre-miRNA-146a causes a mismatch in the miR-146a precursor, with lower expression of the mature form and activation of a toll-like receptor (TLR) signaling pathway involved in stroke risk [13, 14]. miRNA-146a could be involved in IS pathogenesis through its influence on tumor necrosis factor receptor-associated factor 6 (TRAF-6) and interleukin-1 receptor-associated kinase 1 (IRAK-1) with consequent inflammatory cytokines release disruption involving tumor necrosis factor (TNF), IL1, IL-8, and IL6 [15].

This study aims to investigate the probable associations of rs2910164 C > G and rs3746444 A > G genetic variants with IS risk. This study clarifies IS molecular basis for early prediction and prevention of related complications. IS represents a significant health issue in our society with its combined great morbidity and mortality. Moreover, it is one of the burdens on economic resources worldwide [16].

Methods

The current investigation involved 100 adult Egyptian patients who had been diagnosed with atherosclerosis for the first time. Patients with cerebral hemorrhage, embolism, trauma, malformations, and transient ischemic attack (TIA) were excluded. They were collected in the period from July 2018 through August 2019, from the stroke unit of the Neurology department, Faculty of Medicine, Cairo University.

The case group presentation was an acute onset of a focal neurological deficit that persisted for more than 24 h. Cerebral infarction was diagnosed by imaging techniques, including computed tomography (CT) and magnetic resonance imaging (MRI). Participants were subjected to questions regarding their history, clinical examination, and laboratory investigations, including blood sugar, lipid profile, protein C, protein S, anti-thrombin III and immunological screening. National Institutes of Health Stroke Scale (NIHSS) was used to stratify stroke severity [17]. NIHSS is composed of

11 neurological markers. Each one is scaled from 0 as usual to 4 for high disability. The sum of scores classifies stroke as follows: 0 for the absence of stroke, 1–4 for minor stroke, 5–15, and 16–20 for moderate and moderate to severe stroke, respectively, while 21–42 score is considered a severe one.

Co-morbidities as risk factors to atherosclerotic stroke, including smoking, hypertension (HTN), Diabetes Mellitus (DM) and Hyperlipidemia, were evaluated for both groups. HTN was diagnosed by either taking antihypertensive medications, having a systolic blood pressure of 140 mmHg or higher, or having a diastolic blood pressure of 90 mmHg or higher [18]. Hyperlipidemia was detected by the existence of anti-hyperlipidemic drugs' administration, hypertriglyceridemia > 150 mg/dL or increased cholesterol > 200 mg/dL. DM is defined by the presence of either antidiabetic treatment, fasting blood sugar equal to or greater than 126 mg/dL, postprandial blood glucose of 200 mg/dL, or A1C 6.5% [19].

CRP was measured by Immunoturbidimetric assay using COBAS e601. It is measured during the first 24 h of stroke with the exclusion of the presence of any associated infectious diseases. CRP level up to 5 mg/L was considered normal.

Ultrasonography assessed the grade of atherosclerotic stenosis in the extra and intracranial vasculature. The Philips iU22 machine with a linear 5–12 MHz high-frequency probe was used for extracranial carotid vessel examination, while the 1–5 MHz low-frequency probe was utilized for trans-cranial assessment. Von Reutern et al. showed the criteria for Extracranial stenosis [20]. 0–40% was considered low grade, 50–60%, and \geq 70% was considered moderate and severe, respectively. Intracranial stenosis was assessed according to the mean flow velocity of large intracranial arteries, considering moderate degree from 50 to 70% while above 70% as severe [21].

The Control group was involved 100 participants, with age, gender, and race matched. They were selected to have more risk factors for atherosclerotic IS but without evidence of any vascular insult. They were recruited from Internal Medicine Outpatient Clinics, Faculty of Medicine, Cairo University.

DNA extraction

For DNA extraction, 3 ml of blood from a peripheral venous access was taken with a tube of ethylene diamine tetra-acetic acid (EDTA). Samples were kept at -20 °C until usage. Genomic Purification Mini Kit: GeneJET™ (Thermo Fisher) was supplied by Life Technologies of catalog number K0781 for DNA extraction accordingly.

DNA amplification

Applied Biosystems 40 × TaqMan[®] Genotyping SNP assay ready-made was used. The context sequence [VIC/FAM] for rs2910164 of miR-146a was CATGGG TTGTGTCAGTGT CAGACCT[C/G]TGAAATTCA GTTCTTCAGCTGGGAT. For rs3746444 of miR-499a, it was ATGTTTAACTCCTCTCCACGTGAAC[G/A] TCACAGCAAGTCTGTGCTGCTTCC.

Master mix final volume was 20 μL; 10 μL TaqMan[®] of genotyping Master Mix (Thermo Fisher), 3.5 μL of extracted DNA, 0.5 μL of SNP assay, and 6 μL distilled water. Delite Real-Time PCR Thermal Cycler (DNA-Technology) amplification program was followed by holding for 10 min at 95 °C, then 40 cycles consisted of denaturation (at 95 °C for 15 s) and annealing/extension for 1 min at 60 °C. Allelic and genotypic discrimination was done according to the plotting of fluorescence signals.

Quality control was performed. It was carried out by repeating the genotyping on ~10% of the samples and checking the genotype scoring separately by two reviewers blind to case–control status.

Statistical analysis

Hardy–Weinberg equilibrium (HWE) was estimated by a goodness-of-fit (χ^2) test. Data were assessed using SPSS version 26 (IBM Corp., Armonk, NY, USA). Data was formulated as mean and standard deviation (SD) for quantitative variables. Categorical data were assessed as frequencies and percentages. Non-parametric Kruskal–Wallis and Mann–Whitney tests were utilized for differentiating the quantitative data. Chi-square test and Exact test were performed for comparing categorical data. Logistic regression was used to find independent variable predictors and determine the odds ratio (OR) with a 95% confidence level. The receiver operating characteristic (ROC) curve was utilized to detect the classifier performance of data. *p* values ≤ 0.05 were considered as significant.

Results

Both groups were matched regarding age, sex, and presence of risk factors of stroke. Demographic data, risk factors, and stroke assessment scores are shown in Table 1.

Genotyping of the studied groups

For rs3746444 A > G, there was a statistically significant difference regarding the genotypic and allelic frequencies between the two groups. The G allele and GG genotype were considered as risk factors for stroke. The combined GG + AG genotypes demonstrated a 2.077

Table 1 Demographic data, Risk factors and stroke assessment scores

Parameters	Cases (n = 100)	Controls (n = 100)	<i>p</i> Value*
<i>Age (years)</i>			
Mean ± SD	54.35 ± 10.58	51.74 ± 9.35	0.066
Range	22–78	22–78	
≤ 40, n (%)	11 (11)	14 (14)	0.521
> 40, n (%)	89 (89)	86 (86)	
<i>Gender, n (%)</i>			
Male	65 (65)	63 (63)	0.768
Female	35 (35)	37 (37)	
<i>Risk factors to stroke, n (%)</i>			
Smoker	41 (41)	48 (48)	0.319
DM	49 (49)	60 (60)	0.118
HTN	61 (61)	60 (60)	0.885
Hyperlipidemia	30 (30)	41 (41)	0.104
<i>NIHSS score, n (%)</i>			
Mild	49 (49)	–	
Moderate	45 (45)		
Severe	6 (6)		
<i>Duplex atherosclerotic scoring</i>			
Normal	21 (21)	–	
Mild	44 (44)		
Moderate	19 (19)		
Severe	16 (16)		
<i>CRP level</i>			
Mean ± SD	18.79 ± 18.21	–	
Range	0.5–66		
Normal ≤ 5 mg/L, n (%)	32 (32)		
Abnormal > 5 mg/L, n (%)	68 (68)		

DM Diabetes Mellitus, HTN hypertension, SD standard deviation, NIHSS National Institutes of Health Stroke Scale

**p* Value ≤ 0.05 is statistically significant

increased risk for stroke incidence (OR = 2.077, 95% CI = 1.181–3.653 and *p* = 0.011) (Table 2).

The rs2910164 C > G, the G allele showed an increased risk for stroke. The combined GG + GC genotypes showed a borderline statistical significance difference than the CC genotype between the two groups, *p* = 0.052 (Table 2).

The frequencies of observed genotypes for both variants were different from the expected, except for rs2910164 C > G in stroke cases that fulfilled HWE (Table 2). The deviation from HWE might be related to sample size, selection criteria, gene flow, or genetic drift [22].

Analysis of allelic combination frequencies of both variants revealed the association of G allele of rs3746444

Table 2 Genotypic and allelic frequencies of rs3746444 A>G and rs2910164 C>G in the studied groups

Parameters	Control (n.100) No (%)	Cases (n.100) No (%)	p Value*	OR	95% CI
rs3746444 A>G					
<i>Genotypes</i>					
GG	18 (18)	48 (48)	<0.001*	3.930	1.990–7.761
AG	26 (26)	14 (14)	0.556	0.794	0.368–1.713
GG+AG	44 (44)	62 (62)	0.011*	2.077	1.181–3.653
AA	56 (56)	38 (38)	Reference		
P_{HWE}	<0.001	<0.001			
<i>Alleles</i>					
G	62 (31)	110 (55)	<0.001*	2.720	1.807–4.096
A	138 (69)	90 (45)	Reference		
rs2910164 C>G					
<i>Genotypes</i>					
GG	31 (31)	39 (39)	0.057	2.053	0.979–4.305
GC	38 (38)	42 (42)	0.109	1.803	0.878–3.705
GG+GC	69 (69)	81 (81)	0.052	1.915	0.995–3.688
CC	31 (31)	19 (19)	Reference		
P_{HWE}	0.0164	0.2113			
<i>Alleles</i>					
G	100 (50)	120 (60)	0.045*	1.500	1.009–2.229
C	100 (50)	80 (40)	Reference		

HWE Hardy–Weinberg equilibrium

*p Value \leq 0.05 is statistically significant

A>G to both alleles of rs2910164 C>G with increased risk to IS occurrence. The combination of A allele of rs3746444 A>G with C allele of rs2910164 C>G was of a protective value to IS incidence (Table 3).

Relations of genotypic frequencies to clinical data

For rs3746444 A>G, there was an increased frequency of HTN in GG genotype than in AG and AA genotypes with $p=0.028$. CRP levels were higher in G containing genotypes ($p<0.001$) (Table 4). Furthermore, Post hoc pairwise comparisons revealed higher CRP levels in GG and GA genotypes than AA genotype, $p<0.001$ and 0.032 respectively (Fig. 1). Using ROC curve, CRP level of 7.5 mg/L was a good classifier of combined GG+AG

genotypes of miR-499a A>G against AA genotype (AUC=0.809, $p<0.001$, sensitivity=82.3% and Specificity=76.3%) (Fig. 2).

No statistically significant difference was found regarding the genotypic frequencies to age, gender, smoking, DM, and hyperlipidemia. Also, no difference was detected regarding NIH score and duplex atherosclerotic scoring (Table 4).

Considering rs2910164 C>G, there was no statistically significant difference regarding the genotypic frequencies to the demographic data, confounding factors to IS, stroke severity scores, and CRP levels, $p>0.05$ (Table 5).

Using multivariate logistic regression analysis after adjusting the confounding risk factors to IS, the combined

Table 3 Allelic combinations of rs3746444 A>G and rs2910164 C>G

rs3746444 A>G	rs2910164 C>G	Control, No (%)	Cases No, (%)	p Value*	OR	95% CI
G	G	39 (19.5)	70 (35)	0.001*	2.223	1.411–3.503
G	C	23 (11.5)	40 (20)	0.021*	1.924	1.104–3.354
A	G	61 (30.5)	50 (25)	0.220	0.760	0.489–1.179
A	C	77 (38.5)	40 (20)	<0.001*	0.399	0.255–0.625

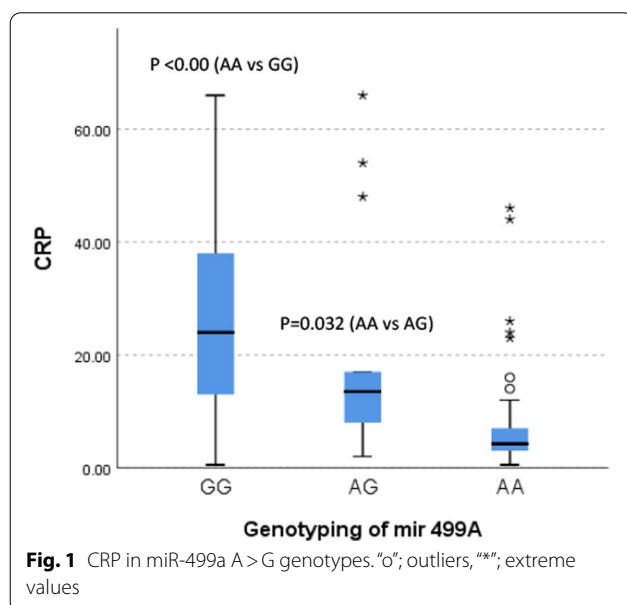
*p Value \leq 0.05 is statistically significant

Table 4 Relations of rs3746444 A > G genotypic frequencies to clinical data

Parameters	rs3746444 A > G				rs3746444 A > G		
	GG N (%)	AG N (%)	AA N (%)	p Value*	GG + AG N (%)	AA N (%)	p Value*
<i>Age (years)</i>							
≤ 40 Years	4 (8.3)	3 (21.4)	4 (10.5)	0.382	7 (11.3)	4 (10.5)	1
> 40 Years	44 (91.7)	11 (78.6)	34 (89.5)		55 (88.7)	34 (89.5)	
Mean ± SD	56.50 ± 10.86	51.43 ± 10.99	54.47 ± 10.90	0.272	55.35 ± 11.01	54.47 ± 10.90	0.541
<i>Gender</i>							
Males	31 (64.6)	11 (78.6)	23 (60.5)	0.528	42 (67.7)	23 (60.5)	0.463
Females	17 (35.4)	3 (21.4)	15 (39.5)		20 (32.3)	15 (39.5)	
<i>Risk factors of stroke</i>							
Smoker	20 (41.7)	6 (42.9)	15 (39.5)	0.968	26 (41.9)	15 (39.5)	0.808
DM	25 (52.1)	4 (28.6)	20 (52.6)	0.257	29 (46.8)	20 (52.6)	0.570
HTN	35 (72.9)	5 (35.7)	21 (55.3)	0.028*	40 (64.5)	21 (55.3)	0.357
Hyperlipidemia	12 (25)	5 (35.7)	13 (34.2)	0.626	17 (27.4)	13 (34.2)	0.472
<i>NIHH score</i>							
Mild	25 (52.1)	7 (50)	17 (44.7)	0.729	32 (51.6)	17 (44.7)	0.338
Moderate	21 (43.8)	7 (50)	17 (44.7)		28 (45.2)	17 (44.7)	
Severe	7 (4.2)	0 (0)	4 (10.5)		2 (3.2)	4 (10.5)	
<i>Duplex atherosclerotic scoring</i>							
Normal	7 (14.6)	5 (35.7)	9 (23.7)	0.335	12 (19.4)	9 (23.7)	0.614
Mild	25 (52.1)	4 (28.6)	15 (39.5)		29 (46.8)	15 (39.5)	
Moderate	11 (22.9)	2 (14.3)	6 (15.8)		13 (21)	6 (15.8)	
Severe	5 (10.4)	3 (21.4)	8 (21.1)		8 (12.9)	8 (21.1)	
<i>CRP levels</i>							
Normal ≤ 5 mg/L	6 (12.5)	3 (21.4)	23 (60.5)	< 0.001*	9 (14.5)	23 (60.5)	< 0.001*
Abnormal > 5 mg/L	42 (87.5)	11 (78.6)	15 (39.5)		53 (85.5)	15 (39.5)	
Mean ± SD	26.60 ± 18.54	20.39 ± 20.11	8.34 ± 10.81	< 0.001*	25.20 ± 18.92	8.34 ± 10.81	< 0.001*

DM Diabetes Mellitus, HTN hypertension, SD standard deviation, NIHH National Institutes of Health Stroke Scale

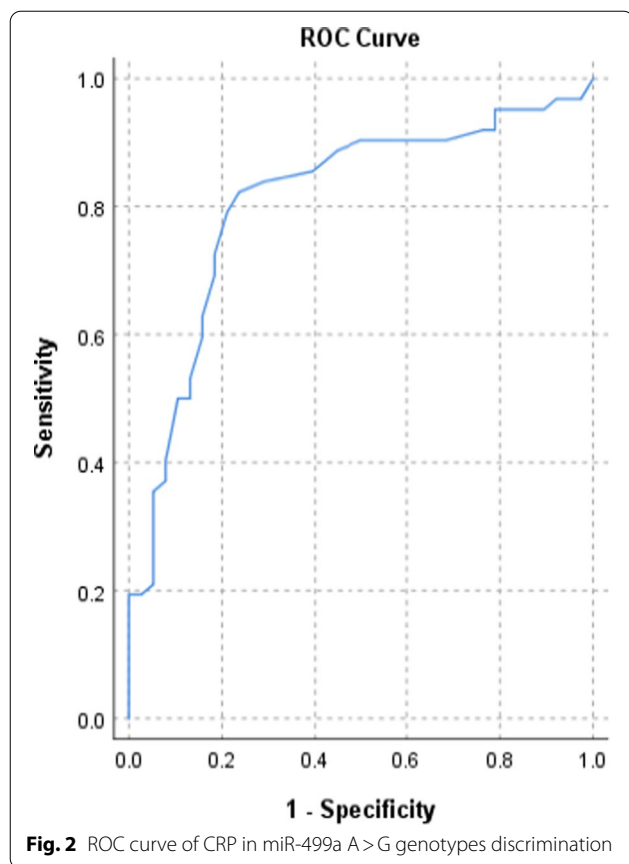
*p Value ≤ 0.05 is statistically significant



variants (GG + AG) of rs3746444 A > G were independent predictors of ischemic stroke, with $p = 0.018$, OR = 1.992 and 95% CI = 1.126–3.523. Unlikely, the rs2910164 C > G variant was not associated with IS ($p = 0.085$, OR = 1.794 and 95% CI = 0.922–3.491).

Discussion

Stroke is a common acquired disorder with the highest mortality rate globally. It is of ischemic or hemorrhagic type [23]. An ischemic type accounts for 85% of the affected stroke cases and is caused by thrombosis, embolism, or hypo-perfusion [2]. Atherosclerosis is the leading cause of IS with multiple environmental and genetic interacting predisposing factors classified as modifiable and non-modifiable risk factors. The modifiable risk factors for atherosclerotic IS include smoking, HTN, hyperlipidemia, and DM [24].



Several susceptibility genes, such as non-modifiable risk factors, are linked to the risk and prognosis of stroke [25]. miRNAs can involve in cell proliferation, apoptosis, and cellular hemostasis [26]. SNVs of pre-miRNAs affect synthesis processing of miRNAs that may involve the action of Drosha and Dicer enzymes, with subsequent function disruption of the mature form of miRNA and emergence of various disorders [27]. Genetic variants in miRNAs or their precursors, pre-miRNAs, play an essential role in IS pathogenesis and its risk factors susceptibility, including HTN, triglyceride disturbance, and DM [28]. The rs2910164 C>G of pre-miRNA-146a and rs3746444 A>G of pre-miRNA-499 are related to several disorders like congenital heart disorder, coronary heart diseases, and malignancies [29]. Both variants play a pivotal role in inflammatory stimulation proposing their effect on stroke risk [10, 30]. Our study confirmed their role in the risk of atherosclerotic IS in the Egyptian population. Both variants were investigated by real-time PCR. As well, CRP levels, as one of the genetic inducing mechanisms to IS, were analyzed by immunoturbidimetry.

Regarding rs3746444 A>G, our study revealed that the G allele and its involving genotypes demonstrated a higher IS risk. Combining the G allele of rs3746444

A>G with either C or G allele of rs2910164 C>G was associated with increased IS susceptibility.

There were increased frequencies of HTN in the GG genotype of rs3746444 A>G than in AG and AA genotypes ($p=0.028$). No statistically significant difference was found regarding the genotypic frequencies to age, gender, smoking, DM, and hyperlipidemia. Also, no difference was detected regarding NIH score and duplex atherosclerotic scoring, $p>0.05$. There was a significant association of the genotypic variants GG, AG of rs3746444 A>G compared to the AA genotype with increased levels of CRP at a cut value of 7.5 mg/L.

The rs3746444 A>G of pre-miRNA-499 is present in 20q11.22 in the 3p strand of the mature form of miRNA. Its association with IS is linked to multiple inflammatory pathways with subsequent thrombosis susceptibility. Several inflammatory mediators are regulated by rs3746444, including IL-2, IL-6, IL-17 receptors, and B, T lymphocytes immune regulatory receptors [31]. The rs3746444 A>G variant affects the expression of the mature form of miR-499a and the control of mRNA functions to influence IS susceptibility [32]. The miR-499a disruption is found to be related to cellular hypoxic state and apoptosis [33].

CRP levels enhance one of the pathways of rs3746444 A>G, inducing inflammation. CRP was found to be raised in G-involved genotypes and could cause hypertension, hyperlipidemia, and insulin resistance with induction of cerebral anoxia [31]. Another possible mechanism of rs3746444 A>G in IS maybe its linkage disequilibrium with other genetic variants involved in stroke pathogenesis [34].

Compatible to our findings, Luo et al. [35] studied both variants in IS versus normal controls with analysis of CRP in the Chinese population. They found that rs3746444 A>G, GG+AG genotypes and G allele demonstrated increased IS risk [$(p=0.027, OR=1.621$ and 95% CI=1.079–2.516) and ($p=0.039, OR=1.455$ and 95% CI=1.019–2.381) respectively]. Also, they detected higher levels of CRP in AG+GG genotypes than found in AA genotypes, $p<0.001$.

In agreement with our results, Liu et al. [36] investigated both variants in IS compared to normal controls by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), and 10% of the results were confirmed by direct sequencing. For rs3746444 A>G, they reported that the G allele showed a significant linkage to IS risk ($p=0.003, OR=1.509, 95\% CI=1.151-1.978$). The GG genotype and AG+GG genotypes had an increased risk to IS than the AA genotype ($p=0.007, OR=1.563, 95\% CI=1.135-2.153$). However, they did not find an association between the genotypic frequencies with NIH scores.

Table 5 Relations of rs2910164 C>G genotypic frequencies to clinical data

Parameters	rs2910164 C>G				rs2910164 C>G		
	GG N (%)	GC N (%)	CC N (%)	p Value*	GG + GC N (%)	CC N (%)	p Value*
<i>Age (years)</i>							
≤40 Years	6 (15.4)	4 (9.5)	1 (5.3)	0.540	10 (12.3)	1 (5.3)	0.685
>40 Years	33 (84.6)	38 (90.5)	18 (94.7)		87.7 (87.7)	18 (94.7)	
Mean ± SD	55.51 ± 11.58	53.71 ± 10.62	56.89 ± 10.38	0.407	54.58 ± 11.06	56.89 ± 10.38	0.426
<i>Gender</i>							
Males	22 (56.4)	30 (71.4)	13 (68.4)	0.345	52 (64.2)	13 (68.4)	0.728
Females	17 (43.6)	12 (28.6)	6 (31.6)		29 (35.8)	6 (31.6)	
<i>Risk factors of stroke</i>							
Smoker	15 (38.5)	20 (47.6)	6 (31.6)	0.458	35 (43.2)	6 (31.6)	0.354
DM	18 (46.2)	25 (59.5)	6 (31.6)	0.117	43 (53.1)	6 (31.6)	0.091
HTN	25 (64.1)	24 (57.1)	12 (63.2)	0.795	49 (60.5)	12 (63.2)	0.830
Hyperlipidemia	13 (33.3)	12 (28.6)	5 (26.3)	0.831	25 (30.9)	5 (26.3)	0.697
<i>NIHSS score</i>							
Mild	21 (53.8)	21 (50)	7 (36.8)	0.732	42 (51.9)	7 (36.8)	0.282
Moderate	16 (41)	19 (45.2)	10 (52.6)		35 (43.2)	10 (52.6)	
Severe	2 (5.1)	2 (4.8)	2 (10.5)		4 (4.9)	2 (10.5)	
<i>Duplex atherosclerotic scoring</i>							
Normal	7 (17.9)	10 (23.8)	4 (21.1)	0.987	17 (21)	4 (21.1)	0.950
Mild	17 (43.6)	18 (42.9)	9 (47.4)		35 (43.2)	9 (47.4)	
Moderate	8 (20.5)	7 (16.7)	4 (21.1)		15 (18.5)	4 (21.1)	
Severe	7 (17.9)	7 (16.7)	2 (10.5)		14 (17.3)	2 (10.5)	
<i>CRP levels</i>							
Normal ≤ 5 mg/L	11 (28.2)	15 (35.7)	6 (31.6)	0.769	26 (32.1)	6 (31.6)	0.965
Abnormal > 5 mg/L	28 (71.8)	27 (64.3)	13 (68.4)		55 (67.9)	13 (68.4)	
Mean ± SD	17.34 ± 16.61	18.90 ± 19.90	21.54 ± 18.11	0.731	18.15 ± 18.29	21.54 ± 18.11	0.431

DM Diabetes Mellitus, HTN hypertension, SD standard deviation, NIHSS National Institutes of Health Stroke Scale

*p Value ≤ 0.05 is statistically significant

According to the current study, Li et al. [37] meta-analysis results for rs3746444 A>G in Chinese individuals demonstrated that the G allele and AG+GG genotypes had increased risk for IS with OR=1.24 and 1.36, respectively.

In contrary to our findings, Liu et al. [36] did not found a reciprocal action between the genetic variants and environmental elements as the AG+GG genotypes increased IS risk more in the young, non-smoker, non-diabetic, non-hyperlipidemic, and non-hypertensive patients with [(OR=1.89, 95% CI=1.22–2.93), (OR=1.88, 95%CI=1.31–2.69), (OR=1.60, 95%CI=1.13–2.25), OR=1.61, 95% CI=1.09–2.38), (OR=2.38, 95% CI=1.44–3.91)] respectively.

Jeon et al. [38] investigated both variants in the Korean population by PCR-RFLP and confirmed their results by direct sequencing. In contrary to our findings, they reported that rs3746444 A>G polymorphism was not distinct between stroke cases and control subjects,

besides its avoidance in association to HTN. However, in agreement with us, they detected no association between its genotypic variants and age, sex, DM, and hyperlipidemia with $p > 0.05$. Zou et al. [5] also reported no association of genotypic and allelic variants of rs3746444 A>G to IS risk.

For rs2910164 C>G, our finding revealed that the G allele carried a higher risk to IS. The combined GG+GC genotypic frequencies showed a borderline statistical significance to CC genotype frequency. No statistically significant difference was detected regarding demographic data, confounding factors to IS, stroke severity assessment scores, and CRP levels in the different genotypic variants. The combination of A allele of rs3746444 A>G with C allele of rs2910164 C>G was of a protective value to IS incidence.

The rs2910164 C>G of pre-miRNA 146a is in chromosome 5 in 3p mature miRNA regions [39]. This variant leads to precursor hairpin mispairing that changes processing

and reduces the production of the mature form [40]. It is related to various human diseases, including vascular damage [39]. Moreover, it regulates TNF- α through the TLR signaling pathway [41]. TNF- α is linked to increased levels of plasminogen activator inhibitor-1 that disrupt the hemostatic balance [42]. It affects atherosclerosis and IS risk by disrupting various inflammatory cytokines release, including IL-6, IL1, monocyte chemotactic protein 1, IL 8, and nuclear factor-Kappa B (NF-kB) [43].

Concomitant to our findings regarding rs2910164 C>G variant, Jeon et al. [38] reported the association of GG+GC and G allele to IS risk [($p=0.051$, OR=1.275 and 95% CI=0.999–1.628) and ($p=0.011$, OR=1.244 and 95% CI=1.051–1.472)] independently. Unlikely, they revealed high demonstration of CC genotype in females, non-hypertensive, and non-diabetic patients [(AOR=1.431 and 95% CI=1.022–2.002), (AOR=1.632 and 95% CI=1.133–2.350) and (AOR=1.355 and 95% CI 1.028–1.785)] respectively.

Consistent with the current study, Zou et al. [5] meta-analysis in Asian populations revealed that the GG variants of rs2910164 C>G associated with IS risk (OR=1.20, 95% CI=1.02–1.42, $p=0.03$). Young et al. [31] demonstrated no association of CRP levels to different genetic variants of rs2910164 C>G, $p=0.436$.

In contrast, Luo et al. [35] did not find any association between genotypic and allelic frequencies of rs2910164 C>G and IS with $p>0.05$. However, in agreement with our results, they reported no association between the frequency of hyperlipidemia in the different genotypic types of both variants.

Contrary to our results, Qu et al. [44] stated that rs2910164 C>G variants were not associated with IS incidence. However, in the follow-up of their patients, they found that the GG genotype was associated with IS recurrence risk, with OR=1.56 and $p=0.016$.

Inconsistent with our finding, Liu et al. [36] and Zhu et al. [45] found no significant difference for rs2910164 C>G genotypic and allelic frequencies between the cases and controls, $p\geq 0.05$ and 0.869 independently. Li et al. [37] meta-analysis study in Chinese ethnic showed that the genotypic and allelic variants of rs2910164 C>G were not associated with IS risk.

The disparity in results between prior studies and our research could be attributable to differences in study method, sample size, patient selection criteria, geographical characteristics, racial factors, and ethnicity.

The limitations of our study were due to foundation absence. It represents in the lacking of confirmatory method to our results that compensated by its verification by sequencing in the previous researches. Larger sample size is recommended in future research for validation of our results.

Conclusion

In conclusion, our study demonstrates the potential function of pre-miRNA variants in stroke incidence. The rs3746444 A>G and rs2910164 C>G variants were associated with IS susceptibility in the Egyptian population. Increased CRP levels in G containing genetic modules of rs3746444 A>G were linked to IS pathogenesis.

Abbreviations

AUC: Area under the curve; CRP: C-reactive protein; CT: Computed tomography; DM: Diabetes Mellitus; EDTA: Ethylene diamine tetra-acetic acid; GWAS: Genome-wide association study; HTN: Hypertension; IL-17RB: Interleukin-17 receptor B; IRAK-1: Interleukin-1 receptor-associated kinase 1; IS: Ischemic stroke; MHz: Megahertz; miRNA: Micro RNA; MRI: Magnetic resonance imaging; mRNA: Messenger RNAs; NF-kB: Nuclear factor-Kappa B; NIHSS: National Institutes of Health Stroke Scale; PCR: Polymerase chain reaction; PCR-RFLP: PCR-restriction fragment length polymorphism; RNAs: Ribonucleic acids; SD: Standard deviation; SNVs: Single-nucleotide variants; ROC: Receiver operating characteristic; TLR: Toll-like receptor; TIA: Transient ischemic attack; TRAF-6: Tumor necrosis factor receptor (TNFR)-associated factor; TRAF-6, TNF: Tumor necrosis factor; UTR: Untranslated regions.

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Authors' contributions

Dr. WM: idea, supervision the work, writing the paper, and contact the journals. DR. NZ: design the study, revision of writing, and validate the final draft. Dr. SAE: revision of practical work, statistical analysis, writing, and approval of final version of paper. Dr. SA: Clinical diagnosis, ultrasound performance, selection of cases, interpretation of clinical data. Dr. KG: collect the data, doing the practical work, practical data interpretation, and writing. The manuscript is the original form of our results that not previously published. All authors read and approved the final manuscript.

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Availability of data and materials

The data of the current research can be obtained through the corresponding author according to reasonable request.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Faculty of Medicine of Cairo University, with the approval number I-370317. It is carried out in accordance with the ethical principles of medical human research in the Declaration of Helsinki. Informed written consent was obtained from each contributor.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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