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Emerging insights into the relationship between pre-microRNA146a rs2910164 gene polymorphism and TNF-α in ischemic stroke

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Abstract

Objectives: This study investigated the association between the pre-miRNA146a C>G rs2910164 polymorphism and serum TNF-α in Egyptian patients with IS.

Methods: A case-control study was conducted on 75 Egyptian cases with IS and 75 sex-matched control subjects aged 57–65 years. Genomic DNA analysis of pre-miRNA146a and TNF-α measurement was performed with real-time PCR and ELISA, respectively.

Results: There was a statistically significant difference between cases of ischemic stroke (IS) and control subjects in pre-miRNA146a rs2910164 GG genotype (p=0.017) and G allele (p=0.005). The pre-miRNA146a rs2910164 is significantly associated with large artery atherosclerosis (LAA) in GG genotypes (p=0.019) and G alleles (p=0.004) compared to control subjects. There was a highly statistically significant increase in TNF-α levels (p<0.001) in IS compared to the control group. There was also a statistically significant increase in TNF-α levels (p=0.001) in GG genotypes in IS.

Conclusions: Our results showed that there was a statistically significant association between pre-miRNA146a rs2910164 GG genotype and susceptibility to IS and LAA. In addition, there was a statistically significant association between pre-miRNA146a rs2910164 GG genotype and TNF-α in IS subjects.

Keywords: ischemic stroke; pre-miRNA146a; rs2910164; TNF-α.

Introduction

Ischemic stroke (IS) is clinically defined as a rapidly progressive syndrome that shows signs and symptoms of a focal or total brain function loss accompanied by cerebral infarction due to inadequate oxygen delivery to the brain [1]. The treatment window for stroke is narrow, and the recurrence rate is very high, making it the primary death cause and a severe economic burden in many areas [2]. The main risk factor for IS is high blood pressure, with other risk factors such as tobacco smoking, obesity, dyslipidemia, and diabetes mellitus [3]. About 80% of strokes are ischemic in origin. The chronic inflammatory disease atherosclerosis characteristics are formed, atherosclerotic plaques and narrowed lumen of the vessel, which is subsequently complicated with IS [4].

Growing evidence suggests that microRNAs (miRNA), including miRNA146a, are involved in the circulatory inflammatory pathways and thrombosis. However, controversial opinions on their roles in stroke were inconclusive [5]. Single nucleotide polymorphisms (SNPs) in microRNAs (miRNAs) result in alteration of miRNA sequence that affects its interaction with messenger RNA causing gene expression dysregulation [6].

Rs2910164 polymorphism presents in chr5:160485411 (GRCh38.p13), C>G [7]. This rs2910164 variant down-regulates the mature miRNA146a expression by altering the nucleotide sequence at the stem region of its precursor [8]. It targets mRNA molecules such as C-reactive protein (CRP),...
interleukin-1 receptor-associated kinase-1 (IRAK1), and tumor necrosis factor-alpha (TNF-α), affecting atherosclerosis inflammatory and vascular injury responses in stroke development. Therefore, miRNA146a’s reduced expression leads to increased atherosclerosis inflammations and vascular damage by elevating the levels of CRP, IRAK1, and TNF-α [9, 10]. Consequently, modulation of the miRNA146 expression in the endothelium can potentially target vascular inflammations in vivo [11].

TNF-α is an essential factor in the inflammatory response in strokes. Overexpression of TNF-α can cause a serious impact on coagulation, endothelial function, and lipid metabolism, contributing to the risk of IS [12].

This work is conducted to study the association of pre-miRNA146a C>G rs2910164 polymorphism and serum TNF-α in an Egyptian group with atherosclerosis-related IS to assess its prediction IS subtype, recurrence, and prognosis.

Materials and methods

This case-control study was conducted on 75 patients with IS (study group) recruited from the neurology department, Kasr Al-ainy, Cairo University, and 75 healthy unrelated volunteers (control group). The diagnosis of IS was made according to American Heart Association guidelines (AHA), 2009. Patients in the current study were included according to the signs and symptoms of ischemic stroke confirmed by CT or MRI. The study group was further subdivided into 19 patients with small vessel disease (SVD) vs. 56 patients with large artery atherosclerosis (LAA) according to the Trial of Orgaran® in Acute Stroke Treatment (TOAST) classification and then recurrent (28 patients) vs. non-recurrent stroke (67 patients). Calculation of the national institute of health stroke scale (NIHSS) for ischemic stroke patients was performed. The interpretations of the results showed that scores >16 represent a high mortality probability, while baseline NIHSS scores <6 represent a high recovery probability [13]. Then, we excluded patients with other different stroke kinds: transient ischemic attack, brain tumors, cerebrovascular malformation, embolic brain infarction, and subarachnoid hemorrhage. Also, we excluded patients with other comorbid diseases such as inflammatory, liver, or renal disease and patients who had cardiac problems such as atrial fibrillation, dilated left ventricle, and infective endocarditis.

The control group was selected with no history of myocardial infarction or cerebrovascular disease. Both groups were subjected to whole personal history taking, present symptoms, signs, and medication history. Laboratory investigations were performed in the form of a lipid profile: total cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL-c), and high-density lipoprotein (HDL-C) carried on Beckman Coulter AU680 (Beckman Coulter, Kraemer Blvd Brea, CA 92821, USA). Immunological tests, including TNF-α concentration in the serum, were carried out using the enzyme-linked immunosorbent assay (ELISA) kit (Glory Science Co., Ltd, Wensan Road, Xihu District, Hangzhou, Zhejiang 310013, P.R China). The purified DNA samples were assessed for dsDNA concentration yield using Nano Drop technologies (ND-1000 UV/Vis, USA). Molecular tests were conducted as follows: extraction of genomic DNA via Gene JET whole blood genomic DNA purification mini kit provided by Thermofisher Scientific company (Waltham, Massachusetts, USA) and detection of C>G polymorphism in pre-miRNA146a gene by Taqman allelic discrimination assay real time PCR using SNP ready-made assay kit C__15946974_10 (carried on Applied Biosystems step one real-time polymerase chain reaction (PCR) system (Applied Biosystems, CA 94404, Foster City, USA)).

Written consent was obtained from each participant before sample collection. The local Ethics Committee approved the study of the clinical and chemical pathology department, Cairo University (Number: I-350316/May 2016).

Statistical analysis was performed using the statistical package for social science (SPSS) software version 17 (SPSS Inc., Chicago, IL, USA). Normality tests were conducted using the Kolmogorov–Smirnov test. Quantitative data were summarized as mean ± SD when normally distributed. The Student’s t-test was used to estimate the difference between the groups when skewed data were expressed as median (min-max), and (25th, 75th). Mann–Whitney U test was used to assess the difference between the two groups. In the case of more than two independent samples, the Kruskal–Wallis test was used to estimate the difference. Qualitative data were summarized as numbers and percentages and compared by Chi-square (X² test). Genotype frequency of the control group was checked for Hardy Weinberg analysis using the X² test and was consistent with the Hardy-Weinberg equilibrium expectations for rs2910164 (HWEP=0.912). The p-value (two-tailed) of less than 0.05 was considered statistically significant [14].

The mean TNF-α level in patients with ischemic stroke was 11.85 ± 16.98 compared with 5.4 ± 10.2 in controls in the study by Yaseen et al. 2014 [15], so the calculated sample size was 150 (75 controls, 75 patients). The sample size was calculated using the Open Epi program (version 3.01, USA) with a confidence level of 95% and power of 80%.

Results

Demographic, clinical, and routine laboratory investigations of the groups involved in this study were summarized in Table 1. Genotype and allele frequency of pre-miRNA146a in ischemic stroke patients and control group concerning their susceptibility, recurrence, and prognosis were summarized in Table 2 and Supplementary Figure 1.

Levels of TNF-α among the studied group are summarized in Table 3, Supplementary Figure 1, and Supplementary Figure 2. Genotype and allele frequency of pre-miRNA146a together with TNF-α in ischemic stroke patients concerning their subtypes (LAA vs. SVD) in comparison to the control group are summarized in Supplementary Table 2, Supplementary Figure 2 and Supplementary Figure 3.

Receiver operating characteristic curve analysis (ROC) was performed for TNF-α to distinguish between IS patients and the control group, and for TNF-α in the GG genotype (variant allele) in IS subjects and other genotypes.

The ROC curve for TNF-α in ischemic stroke and the control group shows 85% sensitivity and 83% specificity at
a cut-off value of 31 pg/mL; the area under the curve (AUC) was 88% (p<0.001) (Figure 1).

While studying the GG genotypes in IS subjects compared to the other genotypes, the ROC curve for TNF-α shows 91% sensitivity and 84% specificity at a cut-off value of 37 pg/mL. The area under the curve was 95% (p<0.001) (Figure 2).

### Discussion

Strokes are the second main death cause globally and one of the leading morbidity causes among the elderly [16]. This study examined the relationship between pre-miRNA146a rs2910164 and TNF-α with susceptibility to IS, IS recurrence, IS severity, and IS subtypes (LAA and SVD).

Pre-miRNA146a was significantly associated with the risk of IS independently of other IS risk factors in GG genotypes (p=0.017) and G alleles (p=0.005). A Chinese study agreed before this association for GG genotypes (p=0.002) and G alleles (p=0.005), also in a Korean study for the GG genotypes (p=0.013) and G alleles (p=0.011) [1, 17]. In light of this, rs2910164 alters the stem structure region of the miRNA146a precursor by substituting C-to-G nucleotide, downregulating the expression of mature miRNA146a. It is assumed that miRNA146a may be protective against atherosclerosis through its ability to reverse the inflammation process [18].

The miRNA146a regulates the interaction between the transcriptional repressor RelB and the TNF-α promoter [19]. As for serum TNF-α levels in the control group and the ischemic stroke group, our study revealed a highly statistically significant difference with higher TNF-α levels between the ischemic stroke cases compared with the control group (p<0.001). These results are consistent with the findings of TNF-α levels (p=0.001) in the study by Yaseen et al. 2014 [15].

An exciting study by Shen and co-workers in 2008 revealed that the G allele is associated with a decrease in the mature form of miRNA146a after measuring it in the peripheral blood [20]. This was in total agreement with our results as the increase in TNF-α promoter [19].

ROC curves were performed to determine the performance of TNF-α in IS groups, either whole subjects vs. control subjects or those carrying GG genotype. In accordance with our results, TNF-α was found to perform as a possible biomarker of acute IS with area under the curve (AUC) of 99% in a study by Fang et al. 2018 [21].

On a specific stroke subtype, there was a statistically significant difference between GG genotype (p=0.019) and G alleles (0.004) in LAA subjects when compared to the control group. However, this rs2910164 did not confer any significant association when comparing GG genotype in ischemic stroke SVD-type subjects and the control group (p=0.52). This observation supported the theory that not all stroke types have the exact genetic causes. LAA is due to the formation of atherosclerotic plaques in medium-sized and large arteries, whereas SVD is due to vascular sclerosis and hyalinization of small perforating arteries due to hypertension [4]. The results of our study appear to be consistent with the theory that a polymorphism in the pre-miRNA146a gene predisposes to large-vessel stroke rather than small-vessel stroke. This may

| Ischemic stroke (n=75) | Controls (n=75) | p-Value |
|------------------------|----------------|---------|
| Age, years             |                |         |
| Female n=69a           |                |         |
| Male n=101a            |                |         |
| Hypertensiona          |                |         |
| Hypertensive (n=61)    |                |         |
| Nonhypertensive (n=89) |                |         |
| Smokinga               |                |         |
| Smoker (n=36)          |                |         |
| Nonsmoker (n=114)      |                |         |
| Diabetesa              |                |         |
| Diabetic (n=41)        |                |         |
| Non-diabetic (n=109)   |                |         |
| Total cholesterol, mg/dL | 204(183–233) | 186(157–215) | 0.003 |
| LDL-C, mg/dL           | 139(109–173)   | 117(88–150)    | 0.001 |
| Triglycerides, mg/dL   | 170(102–206)   | 143(100–201)   | 0.28  |
| HDL-C, mg/dL           | 32(27–40)      | 35(30–44)      | 0.075 |

Data are presented as median (25th-75th). aData are expressed as number (percentage). HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Table 1: Demographic data, clinical data and routine laboratory investigations of the studied groups.
Table 2: Genotype, and allele frequency of pre-miRNA-46a in ischemic stroke patients regarding their susceptibility, recurrence and prognosis) and control group.

| Risk of IS | CC n (%) | G Allele n (%) | G allele n (%) |
|-----------|----------|----------------|---------------|
| Control   | 19(25.4%) | 35/46.6%       | 12/16.1%      |
| IS cases  | 14(21.4%) | 14/20.5%       | 21/65.5%      |

Data are expressed as (n) number (percentage), aGG p-value vs. CC, bCG vs. CC.

Table 3: TNF-α among the studied group.

|          | Ischemic stroke group (n=75) | Control group (n=75) | p-Value |
|----------|------------------------------|-----------------------|---------|
| TNF-α pg/mL | 60(43–79)                      | 15(12–30)            | <0.001  |

Data are presented as median (25th–75th quartiles). TNF-α, tumor necrosis factor alpha.

Figure 1: ROC curve of TNF-α to discriminate ischemic stroke from the control group.

ROC, receiver operating characteristic curve; TNF-α, tumor necrosis factor.

Figure 2: ROC curve of TNF-α to discriminate ischemic stroke subjects with GG genotype vs. the other genotypes.

ROC, receiver operating characteristic curve; TNF-α, tumor necrosis factor; CC, wild allele; GG, variant allele.
be explained by the role of the pre-miRNA146a polymorphism in atherosclerotic inflammation.

A study performed on a Korean population revealed a borderline association between GG genotypes and LAA (p=0.049) [1]. On the other hand, a study by Zhu et al. stated that the C allele of rs2910164 carriers is more likely to develop a large-artery atherosclerotic stroke than those with a different allele [4].

The discrepancy in the results between various studies might be because of variations in genotypes or the differences between geographical and ethnic groups. Furthermore, the selection criteria of the patients may be different. The study at hand excluded any patient who had causes of IS rather than the presence of atherosclerosis in cerebral blood vessels and ensured that ischemic stroke is not of embolic origin. An in-depth examination of serum TNF-α level concerning the type of vessel involved in IS revealed that TNF-α level did not correlate with infarct size. Our findings are consistent with the previously reported studies [22, 23].

In ischemic stroke recurrence and prognosis: almost equal increments were detected in the GG genotype and G alleles among the recurrent group, 50% of subjects with GG genotype compared to the non-recurrent group, 45% subjects, but this did not reach any statistically significant difference (p=0.54). On relying on NIHSS scaling for ischemic stroke prognosis, similar results were found when comparing GG genotypes in subjects with mild and moderate stroke 42.3% vs. subjects with severe stroke 62.5% as there was no statistically significant difference (p=0.25), however; the GG genotype was more pronounced in the severe group. This was in total contradiction to Qu et al. 2016 who stated that there was no significant association between the pre-miRNA146a polymorphism and ischemic stroke incidence but were significantly associated with ischemic stroke prognosis (p=0.002) and stroke recurrence (p=0.014) in the Chinese population [2]. These different findings could reflect ethnic variations and limitations of sample sizes.

Our study has several limitations: First, the small sample size of this study, so statistical analysis should be taken with caution. In addition, the generalizability of our results to a broader geographic context may be limited because of differences between countries in health systems, lifestyle, environment, and genetics. Finally, we did not measure miRNA146a expression in peripheral blood.

**Recommendations**

Suppose future works on large sample sizes and different ethnic groups confirm this association. In that case, incorporating genotyping of pre-miR146a in predicting the risk of ischemic stroke in highly susceptible individuals with other risk factors together with TNF-α should be considered a severe addition of critical care to reduce the burden of this disabling and fatal condition. Suspicion of the IS subtype should also be increased with pre-miR146a GG genotype with an elevated TNF-α. It can be used as adding value to the genetic background of IS that can be used as a target for therapeutic interventions.

**Conclusions**

The data of this study suggested that the GG genotype and G allele of pre-miRNA146a rs2910164 are contributed to the increased risk of ischemic stroke and LAA ischemic stroke among Egyptians. This notion is suggested to be through upregulation of TNF-α, which has a significant role in the inflammatory process involved in the pathogenesis of IS. Also, this study indicated that there was an association between rs2910164 and LAA. On the other hand, we did not find any associated polymorphism with ischemic stroke progression or recurrence.

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**Competing interests:** Authors state no conflict of interest.

**Informed consent:** Informed written consent was obtained from all individuals included in this study.

**Ethical approval:** The research related to human use has complied with all the relevant national regulation, institutional policies, and in accordance with all the tenants of the Helesinki Declaration, and has approved by the Local Ethical Committee of Clinical and Chemical Pathology Department and Kasr al einy ethical committee research in April 2017, approved number is: I-350316

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