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Prognostic utility of MicroRNA-221 and interleukin-6 in cerebral ischemic stroke

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Abstract

Cerebral ischemic stroke has a significant mortality rate and persistent impairment. The initial diagnosis of stroke occurs by magnetic resonance imaging and computed tomography. There is a strong need for more accessible, less expensive, and non-invasive methods besides the neuroimaging methods. MicroRNAs (miRNAs) are critical regulators for ischemic stroke as they are involved in stroke pathophysiology. The goal of the current study was to determine whether microRNA-221 (miR-221) could be used as a diagnostic biomarker for patients with ischemic stroke, and whether it can serve as a promising indicator of the disease severity especially if combined with interleukin-6 (IL-6). The study included 90 subjects, 45 cerebral ischemic stroke patients and 45 controls. MiR-221 was evaluated by quantitative real-time polymerase chain reaction (q-PCR) and IL-6 by enzyme-linked immunosorbent assay (ELISA). Our study results revealed that the serum miR-221 level was significantly reduced in cerebral ischemic stroke patients when compared to the control group (p<0.0001). In addition, serum miR-221 showed a significant negative correlation with cerebral stroke severity (p<0.0001), whereas serum IL-6 showed a significant positive correlation with cerebral stroke severity (p < 0.0001). We also analyzed the receiver operator characteristic (ROC) curve and found that area under the ROC curve (AUC) for severity of ischemic stroke by miR-221 was 0.97 (95% confidence interval 0.93–1, p<0.001). Notably, the combination of serum miR-221 with IL-6 for prediction of ischemic stroke severity showed both increased sensitivity/specificity (AUC=0.99, 95% confidence interval 0.96-1, p<0.001) than miR-221 alone. We concluded that miR-221 constituted a non-invasive, sensitive, and specific biomarker that could be used for diagnosis of ischemic stroke and for prediction of its severity.

Keywords: cerebral ischemic stroke, microRNA-221, interleukin-6, severity.

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Introduction

Cerebral stroke is a major cause for disability and death worldwide.[1] Egypt has the greatest incidence rate of stroke in the Middle East. In 2018, epidemiological research found that the prevalence of ischemic stroke was 963 per 100,000 Egyptians.[2] Cardiac embolism, cerebral microangiopathy, extracranial and intracranial arteriosclerosis may have contributed to stroke.[3] Strokes could be divided into ischemic and hemorrhagic strokes.[4]

Imaging techniques as magnetic resonance imaging and computed tomography are pricy and not available in all hospitals in countries with low income, they cannot be the sole method used for stroke diagnosis. Blood biomarkers are therefore urgently required for the diagnosis and assessment of severity of ischemic stroke.[5]

Micro-RNAs (MiRNAs) have a great role in controlling the expression of genes. MiRNAs may function as trustworthy markers for risk assessment, and diagnosis, in addition to the prognosis of ischemic stroke. They play significant roles in critical pathways linked to the etiology of stroke, such as inflammation, energy failure, and cell death.[6] Some miRNAs are engaged in biological processes which play essential roles in ischemic stroke pathogenesis, such as neurogenesis, angiogenesis, and neuronal death.[7] In addition, some miRNAs may also have a protective effect on the cerebrovascular system following an ischemic stroke.[6]

In cerebral stroke, pro-inflammatory cytokines are secreted by activated microglia and astrocytes[8]. It has been hypothesized that tumor necrosis factor-α, interleukin-1 (IL-1) and IL-6 influence the prognosis of stroke patients.[9] It is important to note that IL-6 is one of the most often targeted molecules in inflammatory etiology. This discovery is especially interesting because IL-6 is a well-known prognostic indicator for atherosclerotic vascular disease.[10]

The goal of this study was to find out the possibility of employing miR-221 as an accurate non-invasive blood-based indicator for diagnosis of ischemic stroke and as a marker for its severity. In addition, this study aimed to find out the correlation of miR-221 with IL-6 in ischemic stroke and if such correlation could be a supporting factor that adds value to assessment of the severity of the disease.

Subjects and Methods

This case-control study included 45 patients (Group A) who were presented with cerebral ischemic stroke at the Emergency Department of Al-Zahraa University Hospital and Kobry El-Koba Military Hospital during the period from September 2021 to May 2022. A comparable number of sex- and age-matched normal volunteers without history of neurological disorders (Group B) served as a control group. Group A was categorized according to the severity into mild ischemic stroke Group A1 (n=17), moderate ischemic stroke Group A2 (n=16), and severe ischemic stroke Group A3 (n=12).

The G power programme was used to determine the sample size (version 3.1.9.2; Germany) [11] setting the power at 80%, the error at 0.05, and the ratio of controls to cases at 1:1. Consequently, 90 involved subjects made up the necessary sample size (45 cases and 45 controls).

Inclusion criteria

Patients with an ischemic stroke were diagnosed clinically and diagnosis was confirmed by computed tomography scan. The National Institutes of Health Stroke Scale (NIHSS) score was used to assess the severity of the ischemic stroke[12] into mild (NIHSS score < 5), moderate (including moderate cases with NIHSS score of 5 to 15 and moderate to severe with NIHSS score of 15 to 21), and severe (NIHSS score > 21).

Exclusion criteria

Patients with haemorrhagic stroke, intracranial tumours, multiple head trauma, systemic haematological diseases, acute contagious serious illnesses, and liver or renal failure were excluded from this study.

Ethical consideration

The Research Ethics Committee of the Faculty of Medicine (for Girls), Al-Azhar University

reviewed and approved and the study protocol (Approval No. 202106908, dated June 2021). Before being enrolled in the study, each study subject provided a written informed consent for participation.

Procedures and assessment

All demographic information of participants were recorded. Routine laboratory investigations which included fasting and post-prandial serum glucose levels, lipid profile and, kidney function tests were carried out using an automated chemistry analyzer (Cobas c311, Roche, Germany), according to the manufacturer's instructions. IL-6 serum level was estimated using commercial enzyme-linked immunosorbent assay (ELISA) kits (Cat no. QK206, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Expression of miRNA-221 using q-PCR:

The extraction of microRNA was done by the miRNeasy Mini kits (cat no. 217004, QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The RNA purity and concentrations were determined by spectrophotometry (NanoDrop2000 Spectrophotometer, Thermo Scientific, USA). The obtained total RNA was subjected to reverse transcription using commercial kits (Cat no. 339340, miRCURY LNA RT Kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions.

The MiR-221 expression level was determined using miRCURY LNA SYBR Green PCR Kits (Cat no. 339345, Qiagen, Hilden, Germany) on RT-PCR (Rotor-gene Q Instrument, Qiagen, Hilden, Germany). The reaction mixture volume was 20 µl containing: 10 µl of SYBR Green master mix, 2 µl of miRNA-221 primer (Primer sequences: Forward primer-5'-AGCTACATTGTCTGCTGGGTTT-3', Cat no. 339306, Qiagen, Hilden, Germany) and cDNA at a concentration of 20 ng/ µl. The level of miRNA was normalized using small nuclear RNA (snRNA)-U6 as an internal control (Sequence of U6: Forward 5'- CTCGCTTCGGCAGCACATA -3'). The thermal cycling conditions used were as follows: an initial activation step at 95 ºC for 2 minutes then 40 cycles, each of a denaturation step at 95 °C for 10 seconds then combined annealing and extension at 56 °C for 60 seconds. Fluorescence was measured in every cycle.

Following the thermal profile, a melting curve was done to ensure specific amplification by monitoring fluorescence while slowly increasing the temperature (from 65–95 ºC). Analysis of melting curve revealed single sharp peak for target miRNA.

q-PCR results calculation: delta cycle threshold (Ct) was calculated via subtracting the Ct value of U6 from the Ct values of miR-221 in all samples. ΔΔCT was calculated by subtracting the average normalized ΔCT of control from all different ΔCT values including the control values, using normalized ΔCT values. $2^{-\Delta\Delta Ct}$ was used to calculate fold changes for relative quantification.

Statistical methods

The statistics package for social science (SPSS Inc., Chicago, Illinois, USA) version 23.0 was used for data analysis. For quantitative data that is normally distributed, the mean, and standard deviation (SD) were assessed. For comparison between two means, the independent-sample t-test was employed. Frequency and percentages were employed to display the qualitative variables, and the Chi-square (X^2) or Fisher exact tests were performed to compare between them. The receiver operator characteristic (ROC) curve analysis was utilized to predict the severity of the stroke. Pearson correlation was employed to detect the strength of association among continuous variables. A p-value of <0.05 was considered significant.

Results

This study involved 90 subjects, divided into 2 groups. Group A (n = 45, ischemic stroke patients), included 24 males and 21 females with an age range of 40-66 years, with a mean age of 53.8 ± 5.8 years. Group B (n = 45) served as age- and sex- matched control group, consisted of 23 males and 22 females, with an age range of 43-60 years and a mean age of 52.2 ± 4.5 years. The clinical data of the study subjects are illustrated in Table 1.

Table 1. Clinical data of the study subjects.

Variables	Ischemic stroke patients (Group A) (n = 45) No (%)	Control subjects (Group B)(n = 45) No (%)	*p*-value
Hypertension			
No	26 (57.8%)	42 (93.3%)	<0.0001
Yes	19 (42.2%)	3 (6.7%)	
Smoking			
No	29 (64.4%)	38 (84.4%)	0.03
Yes	16 (35.6%)	7 (15.6%)	
Diabetes Mellitus			
No	2 (4.4%)	0 (0%)	NS
Yes	43 (95.6%)	45 (100%)	
Body Mass Index (Kg/m^2)			
Mean± SD	29.4±2.9	23.8±1.3	<0.0001
(Minimum-Maximum)	(25-37)	(21-26)	

P > 0.05 is not significant (NS).

The results of the biochemical parameters showed a significant increase of post-prandial blood glucose, creatinine, and lipid profile (p <0.0001) between patients and the control group (Table 2). There was a significant rise in IL-6 and a significant decline in serum miR-221 in patients when compared with the controls (Table 3).

Table 2. Biochemical parameters of the studied groups.

Variables	Ischemic stroke patients (Group A) (n = 45) Mean ±SD (Minimum-Maximum)	Control subjects (Group B) (n = 45) Mean ±SD (Minimum-Maximum)	*p*-value
Fasting blood glucose (mg/dl)	177.9±36 (88-260)	86.1±10.9 (65-107)	<0.0001
Post prandial blood sugar (mg/dl)	272.6±46.4 (103-330)	112.9±9 (95-130)	<0.0001
Urea (mg/dl)	28.3±6.8 (13.2-54)	29.03±4.9 (20.5-40.5)	NS
Creatinine (mg/dl)	0.97±0.3 (0.5-1.9)	0.81±0.2 (0.5-1.3)	0.006
Cholesterol (mg/dl)	199.4±49.9 (98-280)	154±25 (110-200)	<0.0001
Triglyceride (mg/dl)	171.1±71.2 (58-290)	116.2±25.1 (37-170)	<0.0001
HDL (mg/dl)	48±9.7 (31-71)	57.2±7.3 (40-68)	<0.0001
LDL (mg/dl)	118.4±40.5 (47-182)	74.9±16.5 (47-99)	<0.0001

P > 0.05 is not significant (NS).

Table 3. Comparison of interleukin-6 (IL-6) and serum miR-221 between patients and the control groups.

Variables	Ischemic stroke patients (Group A) (n = 45) Mean ±SD (Minimum-Maximum)	Control subjects (Group B) (n = 45) Mean ±SD (Minimum-Maximum)	*p*-value
IL-6 (ng/L)	310.7±110.6 (155-522)	34.2±14.3 (13-62)	<0.0001
Serum miR-221	0.62±0.4 (0.11-1.69)	1.22±0.2 (0.84-1.69)	<0.0001

P ≤ 0.05 is significant. IL-6: Interleukin 6, miR-221: microRNA-221.

The patient subgroups had significantly different levels of IL-6 as the severe cases had the highest level and mild ones had the lowest level (p < 0.001). However, serum miR-221 levels varied significantly amongst patient subgroups being at the lowest level in severe cases and highest in mild ones (Table 4 and Figure 1).

Table 4. Comparison of the studied markers among ischemic stroke patient subgroups.

Studied parameters	Ischemic stroke patients			*p*-value
	Mild (n=17) Mean ±SD	Moderate (n=16) Mean ±SD	Severe (n=12) Mean ±SD	
IL-6 (ng/L)	208.8±49.8	326.2±77.8	434.3±63.6	<0.0001
	p_1 < 0.001; p_2 < 0.001; p_3< 0.001			
Serum miR-221	0.98±0.36	0.53±0.13	0.24±0.1	<0.0001
	p_1 < 0.001; p_2 < 0.001; p_3 = 0.003			

(p_1) represents the difference between mild and moderate. (p_2) represents the difference between mild and severe. (p_3) represents the difference between moderate and severe. *P* ≤ 0.05 is significant.

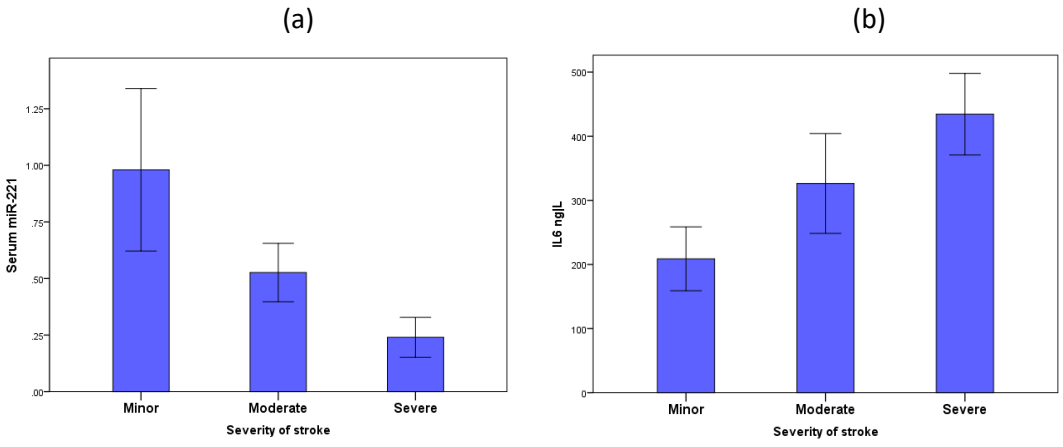

Figure 1. Association between ischemic stroke disease severity and studied diagnostic markers.
(a) Association between serum miR-221 and disease severity according to the NIHSS score.
(b) Association between serum IL-6 and disease severity according to the NIHSS score.

There was a significant negative correlation between serum miR-221 with NIHSS score and IL-6 while there was a significant positive correlation with the grade of weakness (r -0.75, *p* <0.0001, r -0.74, *p* <0.0001 and r 0.79, *p* <0.001, respectively).

According to IL-6, there was a significant positive correlation with NIHSS score and a significant negative correlation with the grade of weakness (r 0.79, *p* <0.0001 and r -0.81, *p* <0.0001, respectively) (Table 5).

Table 5. Correlation between miR-221 and IL-6 in stroke patients with the clinical and studied biochemical parameters.

Different parameters	Serum miR-221		IL-6 (ng/L)	
	r	*p*-value	r	*p*-value
Fasting blood glucose (mg/dL)	-0.31	0.04*	0.34	0.02*
Post prandial blood glucose (mg/dL)	-0.41	0.005*	0.47	0.001*
Urea (mg/dL)	0.06	NS	0.09	NS
Creatinine (mg/dL)	-0.01	NS	0.23	NS
Cholesterol (mg/dL)	0.09	NS	-0.14	NS
Triglyceride (mg/dL)	0.15	NS	-0.04	NS
HDL (mg/dL)	-0.11	NS	0.05	NS
LDL (mg/dL)	-0.05	NS	-0.01	NS
GCS score	0.67	<0.0001	-0.74	<0.0001
NIHSS	-0.75	<0.0001	0.79	<0.0001
Grade of weakness	0.79	0.001	-0.81	<0.0001
IL-6(ng/L)	-0.74	<0.0001	-----	-----

HDL: high-density lipoprotein, LDL: low-density lipoprotein, GCS: Glasgow Coma Scale, NNIHSS: National Institutes of Health Stroke Scale, IL-6: Interleukin-6, miR-221: microRNA-221. *P* > 0.05 is not significant (NS).

Regarding the accuracy of serum miR-221 as a diagnostic marker for acute cerebral ischemic stroke, we analyzed the ROC curve which revealed a cut-off point of serum miR-221as 0.96, with sensitivity and specificity were 91% and 82%, respectively (Table 6).

Table 6. The receiver operating characteristic (ROC) curve analysis for serum miR-221 to differentiate between ischemic stroke cases and the control group.

	Cut off point	*p*-value	Sensitivity	Specificity	Area under the curve (AUC)	95% CI
Serum miR-221	0.96	<0.001	91%	82%	0.91	0.84-0.98

P ≤ 0.05 is significant.

With regard to the diagnostic accuracy of serum miR-221 as a marker of acute ischemic stroke severity, the ROC curve revealed that at a cut-off point of 0.65, with area under the curve (AUC) of 0.97, the sensitivity and specificity for discrimination of mild cases (score <5) from moderate and severe ones (score >5) were 92.9% and 89%, respectively.

To assess the accuracy of the prediction value of serum miR-221 combined with IL-6, the ROC curve with AUC of 0.99 showed both higher sensitivity and specificity of 96.4% and 89%, respectively (Table 7).

Table 7. The ROC curve analysis for the studied diagnostic parameters (serum miR-221 and IL-6 to differentiate between ischemic stroke mild cases and non-mild cases (moderate & severe).

	p-value	Sensitivity	Specificity	Area under the curve (AUC)	95% CI
Serum miR-221	<0.001	92.9	89%	0.97	0.93–1
Combination of Serum miR-221 and IL-6	<0.001	96.4%	89%	0.99	0.96-1

P ≤ 0.05 is significant.

Discussion

Ischemic stroke represents the most prevalent brain disorder worldwide and a major factor in both mortality and morbidity[13], in addition to a significant chance of lifelong impairment[14]. Previous reports showed that dysregulations in microRNA expression were observed as an intermediate process in neuronal cell death cascade following ischemic brain injury, which was mediated by inflammation and oxidative stress[15]. The role of microRNAs in ischemic stroke seems to occur even before the start of the ischemic injury itself. It has been hypothesized that some microRNAs contribute to the emergence and progression of ischemic stroke [16,17]. Therefore, this study aimed to assess the miR-221 expression in ischemic stroke patients and to demonstrate the possible correlation of the marker with disease severity.

Our study revealed that significantly reduced levels of miR-221 were found in cerebral ischemic stroke patients compared with controls. Also, the ROC curve revealed that miR-221 was a specific and sensitive marker for discrimination between ischemic stroke patients and the control group and hence it may be considered as a diagnostic marker for this disease.

Furthermore, serum miR-221 was strongly negatively correlated with the NIHSS score and the degree of weakness. In addition, the serum miR-221 level was significantly different between mild, moderate, and severe cases being lowest in severe cases and highest in mild cases. Also, the ROC curve analysis revealed that serum level of miR-221 alone was a sensitive and specific marker for the prediction of severity. Furthermore, combining the two markers, serum level of miR-221 with serum IL-6, increased the accuracy of the prediction of the disease outcome and the test specificity and sensitivity.

On the contrary, IL-6 levels in stroke patients were considerably greater than those of controls. There was a significant positive correlation between IL-6 and NIHSS and the degree of weakness. In addition, a negative correlation between IL-6 and serum miR-221was found. Also, IL-6 increased the sensitivity and specificity for determination of the severity of ischemic stroke if it was combined with miR-221, therefore it adds to the prediction of the disease course.

Some studies discussed the expression of miR-221 in ischemic stroke patients but up to our knowledge they did not correlate it with the severity of the disease or the clinical outcome. According to a cohort study by Tsai et al, 2013 [18], the level of serum miR-221 is a novel, reliable biomarker for predicting atherosclerosis and stroke being a good risk factor for patients who will develop a stroke. Findings of a study by Jia et al, 2015[19] agreed with our data, as they reported that serum miR-221 levels in ischemic stroke patients were significantly diminished compared to the control group and negatively correlated with serum IL-6. Also, this finding agreed with that of Wang et al, 2017[20] who revealed that circulating serum miRNA-221 level was substantially lower in ischemic stroke patients than control subjects, and they suggested that this marker could be a potential biomarker for diagnosis of ischemic stroke. In

addition, Peng et al, 2020[21] showed that there was downregulation in serum miR-221 in cerebral ischemic stroke and this microRNA regulated the phosphatidylinositol-3-kinase/anaplastic lymphoma kinase (PI3K/AKT) pathway and hence it may promote angiogenesis.

Previous studies disclosed data consistent with our results regarding IL-6 such as Vila et al, 2000[22] reported that baseline IL-6 levels were raised in patients with acute ischemic stroke and early neurological impairment therefore, it may be considered as an important predictor of early clinical deterioration. In the same line, Smith et al, 2004[23] reported that plasma IL-6 levels were significantly linked with the size of the infarct in ischemic stroke patients, the severity, and with the clinical outcome. Also, data by Waje-Andreassen et al, 2005[24] agreed with ours and revealed that IL-6 levels were shown to be greater in patients with acute ischemic stroke when compared to control subjects and to be correlated with the volume of stroke and hence it may be a potent indicator of the neurological deficit severity. Our results agreed with those of Chen et al, 2018[25] who found that serum IL-6 levels were noticeably higher in ischemic stroke patients compared to the control group.

The limitations of the present study included that it did not include follow up of the patients, determination of the markers throughout the course of the disease and studying the effect of the serum miR-221 on the prognosis of the disease.

In conclusion, according to our study findings, serum level of miR-221 may be considered a sensitive and specific marker for diagnosis and for assessing the severity of ischemic stroke especially if combined with IL-6. Such markers could be recommended as diagnostic tools and early predictive markers for the severity of the ischemic stroke disease.

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Author Contributions

WAE and ARM; prepared and approved the entire study protocol, operated the lab experiments, wrote the first draft, had it reviewed, and had it edited. RE, AEE, SME, and FME; guidance for sample collection in accordance with the inclusion criteria, revised clinical information, and patient classification. AAE; interpreted the data and analyzed it statistically and extensively edited the paper. NEI; shared in writing of the first draft and extensively edited the paper and shared in the laboratory experiments. AMA: revised clinical data, centrifugation, and storage of the samples, and shared in the laboratory experiments. All authors have read, revise, and approved the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The Research Ethics Committee of the Faculty of Medicine (for Girls), Al-Azhar University reviewed and approved and the study protocol (Approval No. 202106908, dated June 2021).

Informed consent

Before being enrolled in the study, each study subject provided a written informed consent for participation.

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