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The clinical value of circulating microRNAs in acute ischemic stroke

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Abstract: Acute ischemic stroke is one of the diseases causing death worldwide. MicroRNAs have pivotal roles in acute ischemic stroke pathogenesis, and their expression dysregulation, sensitivity and circulating stability could promote them as diagnostic biomarkers. So, this study aimed to examine the expression fold change and diagnostic accuracy potential of miR-601 and miR-760. The expression pattern of miR-601 and miR-760 in the serum of the 70 acute ischemic stroke patients in comparison to 25 age-matched control subjects was estimated using quantitative real time polymerase chain reaction. The receiver operator curve analysis was performed to determine the diagnostic potential of the studied microRNAs. Down-expression of miR-601 and miR-760 was found (94.8%; -1.16 ±1.05; 45; 75.8%; -0.525±0.936, respectively), the diagnostic accuracy was higher for miR-601 (AUC = 0.859), while the there was no diagnostic accuracy for miR-760. On comparing the studied miRNAs, miR-601 showed the best accuracy than miR-760 regarding the diagnosis of stroke.

Keywords: AIS; miRNA; miR-601; miR-760, Biomarker.

1. INTRODUCTION

Stroke is one of the diseases that causes long term disability worldwide. It is caused due to disruption of blood supply to brain resulting in a disruption in supply of oxygen and nutrients to the brain. Stroke may lead to the death of the brain or permanent damage for the brain[1]. Kumar et al., showed that worldwide, stroke is the secondly most common cause of mortality [2]. Ischemic stroke almost represents 87% of strokes incidents [3]. The incidence rate of stroke in Egypt per year is 137,000 to 250,000 as well as being one of the main reasons of mortality here [4].

All patients having stroke symptoms should be investigated rapidly by brain imaging techniques and the most widely used technique is contrast MRI due to its high sensitivity in identifying small areas of infarction and distinguishing hemorrhage [5]. However, new biological biomarkers are needed for earlier detection and improving diagnosis of AIS.

MicroRNAs (miRNAs) are a group of small non-coding RNAs consisting of 18-25 nucleotides length binding to target mRNAs leading to mRNA degradation or translational suppression. Since their discovery in C. elegans in 1993 it has been shown that miRNAs are responsible for regulating protein expression at the post-transcriptional level [6-8].

Previous evidence and studies showed a correlation between different miRNAs and stroke [9]. It was showed previously that there was an association between elevation of miR-155, miR-107, miR-128b levels and AIS. Consequently, they have a diagnostic value [10, 11]. It is important to explore new targets for emphasizing the diagnostic efficiency of circulating miRNAs in AIS sera, and that is why we choose miR-601 and miR-760.

The current available literature about miR-601 and miR-760 are limited, but both have been turned out to be downregulated with good diagnostic and prognostic values in colorectal cancer [12, 13]. However, they have not been investigated in AIS.

The current study aimed to examine for the first time the expression pattern and the diagnostic potential of miRNAs 601 & 760 in AIS patients' sera.

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2. METHODS

Our study was a retrospective pilot observational study enrolled 95 subjects, 70 of them were AIS patients admitted to the emergency department of Kasr El-Ainy hospital, Cairo University, Egypt, with stroke suggestive symptoms and their diagnosis was confirmed by contrast MRI performed by 3.0 Tesla whole body imaging system with augmented clinical decision, while the other 25 subjects were apparently healthy control subjects. Patients with history of stroke, intracranial hemorrhage, neuropsychological disorders, peripheral vascular disease and myocardial infarction were excluded. The protocol of this study was approved according to the ethical committee of Kasr El-Ainy hospital and all enrolled subjects or the corresponding relatives for AIS patients wrote informed consents.

BD vacutainer system was used for withdrawal of 5 mL peripheral venous blood samples from all subjects. Serum separator tubes were used for serum separation, where blood was left for 15 minutes to clot, and then centrifuged for 10 minutes at 4000 rpm resulting in isolates sera which is followed by storage at -80 °c until being analyzed.

2.1. Isolation of total serum RNA, including microRNAs:

miRNeasy Mini Kit (Cat. No. 217004; Qiagen, Germany) containing lysis reagent (phenol/guanidinethiocyanate) and silica membrane–based purification of total RNA is used for extraction of total RNA from serum sample. The isolation started with addition of 200 µL serum sample to 1000 µL of Qiazol lysis reagent at room temperature for 5 minutes and then 200 µL chloroform was added into the denaturized serum to separate the lysate into aqueous and organic phase. The tubes were mixed well by vortexing for 15 seconds followed by centrifugation for 15 minutes at 14000 rpm at 4 °C. 900 µL of ethanol was applied to the extracted aqueous layer (approximately 600 µL) and mixed by pipetting up and down several times. 700 µL of the mix was applied into RNeasy mini spin columns then centrifuged at 14000 rpm for 15 seconds, the flow-through was then thrown away. To wash the mini spin column, two buffer solutions RWT and RPE were used consecutively and then centrifuged at 14000 rpm for 15 seconds at room temperature. To make sure the spin columns are free from ethanol and dry before elution, they were placed in 2 ml collecting tubes and centrifuged at 14000 rpm for 2 minutes. Finally, to elute Silica-bound RNA, 50 µL of RNase free water was added onto mini spin column and centrifuged at 14000 rpm. Two portions are brought from the division of eluted RNA, 5 µL for NanoDrop spectrophotometer-based RNA quantitation and purity assessment while the reminder was stored at -80 °C to be used in the step of RNA reverse transcription.

2.2 Determining the quantity of total RNA, including miRNAs:

Quantitation of RNA and purity assessment was done by using NanoDrop® (ND)-1000 spectrophotometer (NanoDrop technologies, Inc. Wilmington, USA). The samples were measured through loading the NanoDrop-1000 with 1µL of samples-extracted RNA and their readings were recorded and calculated according to Beer-Lambert's law. The concentration of the nucleic acid in the sample (A260 = 1 = 44 µg/mL) was measured by absorbance at 260.

2.3 Complementary DNA (cDNA) synthesis from the extracted miRNAs:

For cDNA Synthesis, miScript® II RT kit (Qiagen, Germany. Cat. No. 218161) was used as a part of miScriptPCR system. The total RNA containing miRNA is used as a starting material for cDNA synthesis. And for the selective RT of mature miRNA into cDNA, the miScriptHiSpec Buffer was used. The miScript reverse transcriptase mix and extracted RNA samples were left to liquify on ice. On the other hand, RNase-free water, 10x miScriptnucleics mix and 5x miScriptHiSpec buffer were allowed to thaw at room temperature followed by preparation of the reverse transcriptase master mix on ice of a total volume 20 µL by using 4 µL miScriptHiSpec buffer (5x), 2 µL miScriptHiSpec buffer (10x), 2 µL miScript reverse transcriptase mix, 7µL RNase free water, 5µL RNA template (Samples-extracted RNA) in addition to 5µL RNA template (Samples-extracted RNA). The previous mix then was incubated for 60 minutes at 37 °C then another incubation for 5 minutes at 95°C and the final cDNA of each sample was stored undiluted at -80°C to be used in the next step.

2.4 Determination of the quantity of purified miRNA using qRT-PCR:

miScript SYBER ® Green PCR kit (Qiagen, Germany. Cat. No. 218073) with its associated protocol was utilized in this step. Primers for miR-601, miR-760 and the housekeeping control SNORD68 were used. 200 µL of RNAse free water was added to the thawed samples to be diluted. 12.5 µL QuantiTect SYBR Green PCR Master Mix (2x), 2.5 µL miScript Universal primer (10x), 2.5 µL miScript Primer assay (10x), 5 µLRNase free water and 2.5 µLcDNA templates were the components of the reaction mix which applied to the Rotor-Gene Q 72-well rotor (Qiagn, USA) to quantify the targeted miRNAs. The amplification cycles were adjusted to be 40 cycles starting with 15 minutes incubation at 95 ^{0}C to initially activate the mix, then 3 phases of reactions: denaturation of DNA at 94 ^{0}C for 15 seconds, annealing at 55 °C for 30 seconds and

extension for 70 °C for 30 seconds. The primers sequences were as follows: miR-601, 5′-GCTCTCCCAACCCTTGT-3′ (forward) and 5′-CUCCUCCAACAAUCCUAGACCATT-3′ (reverse), miR-760, 5′-TCAATCCACCAGAGCATGGATAT-3′ (forward) and 5′-CTCTACAGCTATATTGCCAGCCA-3′ (reverse) and SNORD68, 5`-CGCGTGATGACATTCTCC-3` (forward) and 5`-GATGGAAAAGGGTTCAAATGT-3` (reverse).

2.5 Results Calculation:

Melting curves were analyzed after completion of qRT-PCR cycles for the validation and confirmation of the specific expression of the targeted miRNAs. Also, cycle threshold (Ct) values were calculated automatically usingRotor-Gene Q software 2.1 (Qiagen). Accordingly, ΔCt method was used for evaluation of the relative expression of SNORD-68 where a substraction of Ct values of SNORD-68 from Ct values of the targeted miRNAs were done for both control and patients' groups followed by calculation of ΔΔCt values. This step is done by subtracting ΔCt values of control group from ΔCt values of the patients group. Finally, the $2^{-\Delta\Delta Ct}$ method is used for calculation of the fold changes (FC) which is the expression ratio or relative quantitation (Rq) for the target miRNAs [14].

2.6 Statistical analysis:

The statistical analysis was carried out, and charts were built using GraphPad Prism 8.02. The values were displayed as mean ± standard deviation (SD), median, number, and percentage. Kolmogrov-Smirnov normality test was used to discover the normal distribution pattern between the data of the groups. The Student's *t* test, Man–Whitney *U* tests were used to compare the difference between two groups, and multiple comparisons between more than two groups were determined by the one-way analysis of variance (ANOVA).

The diagnostic accuracy, cutoff values, sensitivity, and specificity for each miRNA were evaluated by the receiver operator characteristic (ROC) curve and the area under the curve (AUC). While Pearson's correlation test was used for the correlation between the expression level of miRNAs and the biochemical investigations of AIS patients. A significant statistical difference is considered if $P<0.05$.

3. RESULTS

3.1 Demographics description and routine biochemical findings

Among the 95 subjects enrolled in the study, 70 of them was AIS male patients aged (59.4 ±8.36, years) with suggestive symptoms and confirmed diagnosis by clinical decision and MRI findings and 25 male age-matched (57.3 ±3.27, years) apparently healthy control subjects. Their characteristics and routine biochemical findings are listed in table 1.

3.2 Studied miRNAs fold changes expression in AIS sera:

The expression pattern of miRNAs was studied in AIS patients' sera: miR-601 and miR-760. Their expression was downregulated (94.8%; -1.16 ±1.05; 45; 75.8%; -0.525±0.936, respectively) as shown in table 2, figure 1 and figure 2. The most consistent expression pattern observed was miR-601.

3.3 The diagnostic accuracy of the examined miRNAs in AIS Patients:

The ROC curve analysis was used to determine the diagnostic efficacy of the studied miRNAs to distinguish between AIS patients and normal subjects as shown in table 3, figure 3 and figure 4.

MiR-601revealed the highest diagnostic accuracy for AIS diagnosis when compared to control subjects (AUC = 0.859) with sensitivity 79.3% while miR-760 showed low diagnostic accuracy (AUC = 0.696) and sensitivity 60 %.

3.4 Correlation coefficient between the investigated miRNAs

To evaluate the strength of linear correlation between the expression of miR-601 and miR-760 in AIS patients with the clinicopathological findings, pearson's linear correlation coefficient was done (listed in table 4). They showed moderate significant positive correlation ($r = 0.508$ at $p < 0.0001$).

Table 1: Demographics description and routine biochemical findings:

	Control Subjects	AIS patients	P-Value
N	25	70	-
Age (range, years)	49 – 62	42-79	0.2328
(mean ± SD, years)	57.3 ±3.27	59.4 ±8.36	
RBG (range, mg/dl)	85.0 - 128	105-398	<0.0001*
(mean ± SD, mg/dl)	103 ± 13.1	218±98	
T.Ch (range, mg/dl)	134 - 202	137-259	0.0020*
(mean ± SD, mg/dl)	180 ± 18.4	196±26.7	
TG (range, mg/dl)	125 - 168	52-219	0.1162

	Control Subjects	AIS patients	P-Value
(mean ± SD, mg/dl))	147 ± 15.5	132±37.5	
LDL-ch (range, mg/dl)	78.0 - 142	72-178	0.0047*
(mean ± SD, mg/dl)	108 ± 5.16	122 ± 23.7	
HDL-ch (range, mg/dl)	30.0 - 76.0	35- 62	0.0411*
(mean ± SD, mg/dl)	50.8 ± 12.8	47± 6.73	
VLDL-ch (range, mg/dl)	19.2 – 42.0	10.4-43.8	<0.0001*
(mean ± SD, mg/dl)	32.2 ± 6.84	26.5±7.49	
Cr. (range, mg/dl)	0.860 – 1.21	0.88-1.50	<0.0001*
(mean ± SD, mg/dl)	1.00 ± 0.113	1.17±0.135	
Urea (range, mg/dl)	18.0 – 43.6	24-51	0.0071*
(mean ± SD, mg/dl)	32.2 ± 8.16	37±7.18	

AIS: Acute ischemic stroke; SD: standard deviation; RBG: Random blood glucose; T.ch: Total cholesterol; TG:Triglyceride; LDL: Low density lipoproyein; HDL: High density lipoprotein; VLDL: Very low density lipoprotein; Cr: Creatinine.

* Significant from control subjects at p< 0.05

Table 2: The expression fold change of serum-selected miRNAs in AIS

MiRNAs	AIS (n= 70)
MiR-601	
Mean ± SD	-1.16 ±1.05
Median (IQR)	-1.68 to -0.525
Range	-4.21 to 1.01
P	<0.0001*
MiR-760	
Mean ± SD	-0.525 ±0.936
Median (IQR)	-1.16 to 0.031
Range	-2.96 to 2.21
P	0.0099*

AIS: Acute ischemic stroke; DM: Diabetes mellitus; HTN: Hypertension; IQR: interquartile range; SD: standard deviation; miRNA: microRNA

* Significant from control subjects at p< 0.05

Table 3: The diagnostic accuracy of the targeted miRNAs in AIS patients from the control subjects:

MiRNAs	Cutoff	AUC	Sn%	Sp%	95% CI	P
MiR-601	< -0.509	0.859	79.3%	84.0%	0.763- 0.956	<0.0001
MiR-760	< -0.297	0.696	60.0%	80.0%	0.585-0.807	0.0047

AUC: area under the curve; Sn: sensitivity; Sp: specificity; CI: confidence interval; miRNA: microRNA

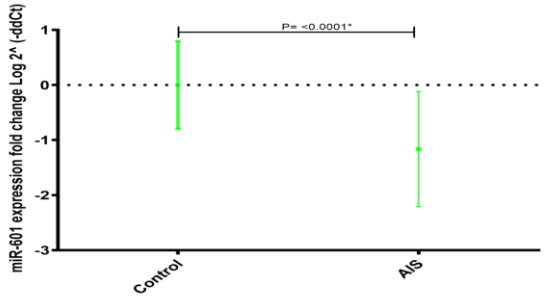

Figure 1. The mean (±SD) relative expression fold change of targeted miRNA-601

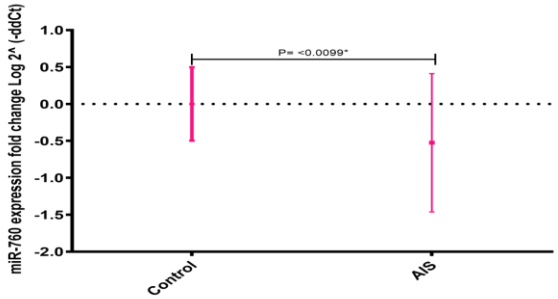

Figure 2. The mean (±SD) relative expression fold change of targeted miRNA-760

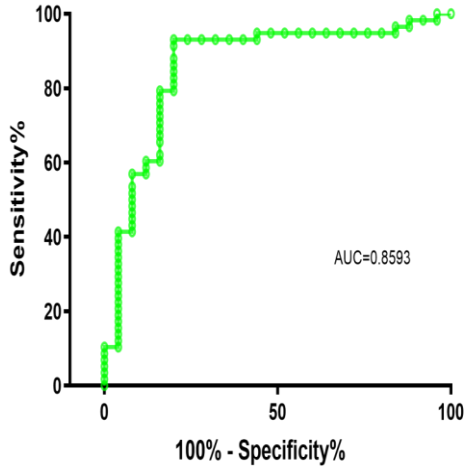

Figure 3. The ROC curve of serum miR-601 to discriminate AIS patients from control subjects

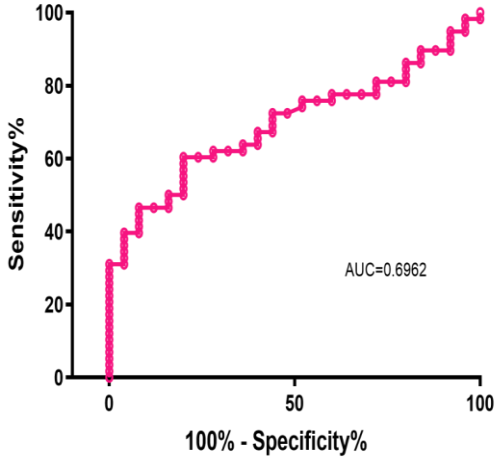

Figure 4. The ROC curve of serum miR-760 to discriminate AIS patients from control subjects

Table 4: Pearson correlation coefficient between the studied miRNAs and patients' characteristics:

Parameters	miR-601		miR-760	
	r	p	r	p
Age	-0.223	0.0918	-0.0517	0.7000
Rbs	0.0802	0.5497	0.116	0.3846
T.ch	0.0237	0.8598	0.244	0.0651
Tg	0.00553	0.9671	0.0563	0.6747
LDL-ch	0.0562	0.6752	0.245	0.0638
HDL-ch	-0.113	0.3988	0.0498	0.7106
VLDL-ch	0.00553	0.9671	0.0563	0.6747
Cr	-0.160	0.2299	-0.103	0.4434
Urea	-0.262	0.0467*	0.0469	0.7268
miR-601	1	-	0.508	<0.0001*
miR-760	0.508	<0.0001*	1	-

*Significant linear correlation at p< 0.05 (two-tailed)

4. DISCUSSION

The diagnosis of AIS can only be done through contrast MRI and there are no current systemic biomarkers for early diagnosis. Recently, investigators have documented that human plasma contains unique miRNAs profiles for specific diseases. Moreover, due to their stability and reproducibility in serum, they have been found to be different noninvasive biomarkers for the diagnosis of multiple disorders [15]. Our study aimed to investigate the expression pattern of miR-601 and miR-760 in AIS patients' sera.

Regarding the studied miRNA-601 in ischemic stroke, no research was done on it. So, it got our interest to determine its expression behavior in-addition to its diagnostic potential in AIS serum. Its Chromosomal location is 9q33.2, within DENND1A (DENN/MADD domain containing 1A) gene. Cell cycle regulation is predicted as its function [16, 17]. Many studies previously showed an apoptotic important role for miR-601 in different types of human cancers where abnormal expression was observed in prostatic cancer, gastric cancer, hepatocellular carcinoma and breast cancer [13, 18-21].

MicroRNA-601 was downregulated with great significant difference from the control group. Considering its diagnostic value and ROC curve analysis, its diagnostic accuracy among the studied miRNAs was (AUC=0.859, P=<0.0001) with Sn=79.3%% and Sp= 84%. In our study, it was the first time to investigate miR-601 in acute ischemic stroke patients. Further experimental models are recommended to be performed to explore the

mechanisms beyond its downregulation in AIS and the molecular mechanisms in pathogenesis.

The second studied miRNA was miR-760. It is located in intron-1 of BCAR3 gene [22]. Also, it is one of the miRNAs that has little attention in AIS research. Regarding its expression, it was downregulated with low significant difference from the control group. Considering its diagnostic value, it showed no diagnostic accuracy (AUC= 0.696, P= 0.0047) with Sn=60% and Sp= 80%. Many previous studies investigate the role of miR-760 in the regulation of cell proliferation, migration, differentiation, and apoptosis where it was mainly downregulated [23, 24] Although, there is no significant results in our study concerning miR-760 but its downregulation also here in AIS patients like its expression in cancer may give a rise to its mechanism on further studies.

It has been found that miR-760 may have a regulatory pathway in pulmonary arterial hypertension (PAH) by regulating toll-like receptor 4 (TLR4) through adjusting its anti-proliferation effect [25].

This study has many limitations to be addressed for further opportunities; Firstly, wider sample size is needed for clearer description of their expression in Egyptian population. Second, wide number of miRNAs screening is recommended to choose the most relevant miRNAs to be later investigated, as the currently investigated miRNAs were selected from the literature. Third, long term monitoring of AIS patients is needed to disclose the change in miRNAs expression in association with AIS symptoms and consequences improvement or declining.

5. CONCLUSIONS

The findings of the present study showed that there were significant differences for both miRNAs between AIS and control subjects although miR-601 show more prominent result and accuracy when compared to miR-760.

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Ethics statement: This study was done according to the Ethics Committee of the faculty of Pharmacy Al-Azhar University, Egypt (permit number:157/2017).

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