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The expression and significance of long noncoding RNA XIST/microRNA-340-5p axis and metabolic reprogramming biomarkers in acute cerebrovascular stroke patients

A cross-sectional study

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Abstract

Stroke represents a worldwide major cause of death and long-term adult disability. Various human diseases pathogenesis, including stroke, are associated with dysregulation of long noncoding RNA (LncRNA) and microRNA (miR). However, their potential role is yet to be elucidated. This work aimed to assess the role of LncRNA X-inactive specific transcript (XIST), miR-340-5p, and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB)3 as peripheral blood biomarkers for acute cerebrovascular stroke diagnosis and severity prediction. This cross-sectional study included 120 participants divided into 3 groups; healthy controls, acute ischemic stroke patients, and acute hemorrhagic stroke patients. XIST, miR-340-5p, and PFKFB3 expression were assessed by RT-qPCR, whereas PFKFB3, hypoxia inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) serum proteins were measured by ELISA. Compared to healthy control, XIST and PFKFB3 mRNA expression were significantly upregulated in stroke patients, with the highest levels in hemorrhagic type, while miR-340-5p expression was significantly downregulated and its lowest level was in hemorrhagic stroke. Serum PFKFB3, HIF-1α, and VEGF levels were significantly elevated in stroke patients with the highest levels in hemorrhagic stroke. These biomarkers correlated with National Institute of Health Stroke Scale (NIHSS). Regression analysis using NIHSS as dependent variable confirmed that PFKFB3 mRNA relative expression was the independent predictor ($\beta = 0.7$, $P = .003$). Receiver operating characteristic analyses revealed that XIST, miR-340-5p, and PFKFB3 mRNA relative expression levels were useful biomarkers discriminating ischemic from hemorrhagic stroke (AUC were 0.99, 0.979, and 0.980, respectively). XIST, miR-340-5p, and PFKFB3 might be involved in acute cerebrovascular stroke pathogenesis and progression providing opportunities for early detection and assessing the severity.

Abbreviations: HDL-C = high-density lipoprotein–cholesterol, HIF-1α = hypoxia inducible factor 1-alpha, INR = International normalized ratio, LDL-C = low-density lipoprotein–cholesterol, LncRNA = long noncoding RNA, miR = microRNA, NIHSS = NIH stroke scale/score, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase, PT = prothrombin time, RBG = random blood glucose, TC = total cholesterol, TG = triacylglycerol, VEGF = vascular endothelial growth factor, XIST = X-inactive specific transcript.

Keywords: cerebrovascular stroke, diagnosis, long noncoding RNA XIST, miR-340-5p, PFKFB3

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1. Introduction

Stroke is an acute cerebrovascular disease of 2 main clinical types: ischemic and hemorrhagic. Ischemic stroke, accounting for around 80% of cases, is triggered by blood flow interruption to the brain, while an abnormal vascular structure or a blood vessel collapse triggers hemorrhagic stroke.[1] Stroke remains a massive health problem, ranking the second cause of death and disability worldwide in 2019, placing heavy burdens on patients' families and society.[2] In Egypt, stroke is also a devastating neurological disorder, responsible for 6.4% of all deaths, coming in as the third cause after heart and liver diseases.[3]

The molecular pathophysiological mechanisms underlying stroke remains unclear, however a large body of evidence suggests that excitotoxicity, mitochondrial dysfunction and oxidative stress are major contributing factors.[4] Hyperacute clinical assessment is still challenging. Hence, further research is significantly important to develop sensitive and reliable blood-based predictors to distinguish an ischemic from hemorrhagic lesions and further provide additional information for pathogenesis understanding and urgent management.[1]

Long noncoding RNAs (LncRNAs) are a category of noncoding RNAs, implicated as key players in both physiological and pathological conditions of various cellular functions. LncRNAs control their target genes expression at epigenomic, transcriptional, or posttranscriptional levels. Besides, lncRNAs can downregulate microRNAs (miRs), serving as molecular sponge.[5] A plenty of dysregulated lncRNAs have drawn wide attention to partake in acute stroke pathology, serving as potential biomarkers for diagnosis, prognosis, progression monitoring, as well as novel potential therapeutic targets.[6]

LncRNA X-inactive specific transcript (XIST) is a major regulator of mammalian X chromosome inactivation. LncRNA XIST is described as an oncogene in several types of cancers by activating a variety of tumorigenic signal pathways involving hypoxia-induced angiogenesis and glycolysis.[7] It was upregulated in diabetic coronary artery disease patients[8] and in ox-LDL treated vascular smooth muscle cells, suggesting it to be a potential target for atherosclerosis-related vascular disease.[9] LncRNA XIST level was also found to increase in atherosclerosis and heart attacks, which are high-risk factors of stroke.[10,11]

MicroRNAs (miRs) are another class of noncoding RNAs, governing gene expression through targeting the mRNA for degradation or translational repression. In the setting of stroke, a growing number of preclinical and clinical studies have revealed an association between certain microRNAs levels and stroke as potential sensitive biomarkers in the diagnosis, as well as therapeutic tools.[12]

Most recently, bioinformatics analysis revealed the interaction between lncRNA XIST and miR-340-5p.[13,14] Recent studies indicated the protective role of miR-340-5p in a variety of cardiovascular and cerebrovascular diseases, including myocardial ischemia–reperfusion injury.[14] MiR-340-5p was elucidated to be a specific regulated target for neural protection in various experimental models.[15,16]

Accumulating evidence implicates metabolic reprogramming as a key driver of the inflammatory response in stroke.[17] Thus, another target has been selected to be investigated, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB)3, a member of the PFKFB family of bi-functional isozymes. PFKFB3 has a crucial role in regulating glycolysis by catalyzing the synthesis of fructose-2, 6-bisphosphate that is a powerful allosteric activator of the key enzyme of glycolysis, 6-phosphofructo-1-kinase. PFKFB3 is ubiquitously expressed in human tissues, with the highest concentration in muscle, fat, kidney, lung, and brain.[18] Overexpression of PFKFB3 was revealed in activated inflammatory cells and numerous tumor cells. PFKFB3-driven glycolysis was also reported in the activation of endothelial cells, while endothelial cell-specific deletion of PFKFB3 reduces pathological angiogenesis.[19]

Despite prior preclinical studies that have demonstrated the role of lncRNA XIST, miR-340-5p, and PFKFB3 in the pathology of ischemic stroke,[20–25] no available data concerning their clinical significance in both types of stroke has yet been studied, to the authors' knowledge. Therefore, this study was directed towards evaluation of the diagnostic value of lncRNA XIST, miR-340-5p, and PFKFB3 in acute cerebrovascular stroke types and their significance in predicting the severity in Egyptian patients.

2. Subjects and methods

2.1. Study population

The current cross-sectional study was conducted in the Departments of Medical Biochemistry, Clinical pathology, Pharmacology, and Neurology, Faculty of Medicine, Tanta University, Tanta, Egypt. For this study, 120 participants were enrolled, categorized into 3 groups, each of 40 as follows: group 1 represented the healthy controls; group 2 represented acute ischemic stroke patients, and group 3 represented acute hemorrhagic stroke patients. The stroke patients were consecutively recruited from the intensive care unit of the neuropsychiatry department, Tanta University Hospital, Tanta, Egypt. The inclusion criteria included those diagnosed with acute stroke (hemorrhagic and ischemic) based on neurological examination and brain imaging (either computed tomography and/or magnetic resonance imaging). The exclusion criteria included patients older than 60 years and younger than 40 years; traumatic brain injury; brain tumor; other brain inflammatory diseases; cerebrovascular malformations; other ischemic diseases; advanced renal, and hepatic insufficiencies; heart failure; chronic infectious diseases; coagulation disorders; autoimmune diseases; and patients who refused to give consent.

The control group was healthy volunteers without a history of stroke, other neurological or systemic diseases and matched the patients' groups in age and sex distribution.

All patients were subjected to thorough history taking and full clinical assessment including general and neurological examination. The severity of stroke was assessed with the NIH Stroke Scale/Score (NIHSS) by experienced neurologists within the first 24 hours after the stroke event. An informed consent was obtained from all individuals included in this study. The study followed the ethical guidelines of Helsinki Declaration, revised in 2008, and was approved by the Research Ethical Committee, Faculty of Medicine, Tanta University, Tanta, Egypt (approval code 36264PR63/1/23).

2.2. Sample collection and biochemical investigations

Within 24 hours of admission, 5 mL of peripheral venous blood were collected from each participant under standard infection control guidelines. Blood samples were divided into 2 parts; 1 part was collected on ethylene diamine tetra acetate (EDTA) for RNA extraction, the other part was drawn into plain tubes for separating serum to determine random blood glucose (RBG), triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and creatinine levels using automated chemistry analyzer (Beckman coulter AU48, Siemens, Kearney, NE, USA). Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald formula. Enzyme-linked immunosorbent assay (ELISA) was used for the assessment of serum hypoxia inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF) levels utilizing ELISA kits (cat no MBS2702488 and MBS355343 respectively, MyBioSource, San Diego). Additionally, serum level of PFKB3 was determined by ELISA kit purchased from FineTest, Wuhan, China (Cat no. EH11044) following the manufacturer's instructions using ELISA

Reader (Stat Fax®2100, Fisher Bioblock Scientific, Illkirch-Graffenstaden, France).

2.3. Quantitative analysis of lncRNA XIST, miR-340-5p, and PFKFB3 mRNA expression by real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the fresh EDTA blood using the Direct-zol™ RNA MiniPrep (cat. no. R2051, ZYMO Research, Irvine) following the manufacturer's instructions. The concentration and quality of RNA were tested using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). Next, the extracted RNA was converted to cDNA by reverse transcriptase using TOPscript™ RT DryMIX (dT18/dN6 plus) (cat. no. RT220, Enzynomics, Daejeon, Republic of Korea) according to manufacturer's instructions. The resulting cDNA was amplified using TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (cat. no. RT500S, Enzynomics, Daejeon, Republic of Korea). Rotor Gene Q5 plex System (Qiagen, Germany) was used for amplification and relative quantitation of lncRNA XIST, miR-340-5p, and PFKFB3 mRNA expression relative to the housekeeping gene using $2^{-\Delta\Delta CT}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for LncRNA XIST and PFKFB3 mRNA expression, while RNU6 was used for miR-340-5p as an endogenous control. Both reference genes were validated for constant expression within the groups.

The primers sequences for the investigated genes were as following: lncRNA XIST Forward: 5'-AGCTCCTCGGACAGCTGTAA-3' and Reverse: 5'-CTCCAG ATAGCTGGCAACC-3', miRNA 340-5p Forward 5'-CGGCGGCTTATAAAGCAA TGAGA-3' and reverse 5'-CCAGTGCAGGGTCCGAGGTAT-3', PFKFB3 Forward: 5'-GTGCCTTAGCTGCCTTGAGA and Reverse: 5'-CCGACTCGATGAAAAACG CC-3', GADPH Forward: 5'-ACAGCCTCAAGATCATCAGC-3' and reverse: 5'-GGTCATGAGTCCTTCCACGAT-3', and RNU6 Forward: 5'-CTCGCTTCGGCAGCACAT-3' and reverse 5'-TTTGCGTGTCATCCTTGCG-3'. Thermal cycling profile was as the manufacturer instructed; 1 cycle of initial activation of DNA polymerase at 95°C for 15 minutes, then repeated 3-step cycling for 45 cycles of (denaturation at 95°C for 10 seconds; annealing at 61°C for 15 seconds and extension at 72°C for 30 seconds). The process was followed by melting curve analysis to ensure the specificity and identity of PCR product. Negative controls were included in each PCR run. Samples were tested blindly and in duplicates.

2.4. Statistical analysis

Statistical package of Social Science (SPSS) version 20 (SPSS Inc., Chicago) was used for data analysis. Data showed normal distribution according to Shapiro–Wilk test. Continuous quantitative data were expressed as mean ± standard deviation (SD) and analyzed applying analysis of variance (ANOVA) test followed by post hoc Tukey test. Bonferroni correction was conducted to adjust the significance level for each pairwise comparison, thus reducing the likelihood of obtaining a false positive (type I error). Qualitative data were represented as frequencies (n) and percentages (%) and analyzed applying chi-square test. *P* values of <.05 were considered statistically significant. Pearson correlation was conducted to evaluate the relation in-between the different variables. Multiple linear regression analysis was used for predicting stroke severity. A receiver operating characteristic (ROC) curve analysis was run for the studied biomarkers to assess the performance of the studied parameters to reach the best compromise in the setting of different types of acute stroke.

The effect size and power of the study were retrospectively checked using SPSS at α-error computed to 0.05. The effect sizes (partial eta^2) were 0.729, 0.819, 0.969, 0.888, 0.967, and 0.935 for HIF-1α, VEGF, PFKFB3 concentration, PFKFB3 expression, miR-340-5p, and lncRNA XIST respectively. The power of the study was 1.0 for all of them.

3. Results

3.1. Demographic, clinical and laboratory data of the studied groups

The study was carried out on 80 stroke patients; 40 patients with ischemic stroke and 40 with hemorrhagic type in addition to 40 healthy controls of matched age and sex. The demographic, clinical, and laboratory data of the participants were presented in Table 1. There were overall statistically significant differences among the studied groups regarding the medical history of diabetes mellitus, hypertension, and ischemic heart disease. Also, comparison of the laboratory findings showed statistically significant differences regarding most routine laboratory data.

3.2. Serum levels of HIF-1α, VEGF, and PFKFB3 in the studied groups

As indicated in Table 2, the mean values of HIF-1α, VEGF, PFKFB3 serum levels were significantly higher in both stroke groups compared with those reported in the control group, where the highest significant values were for hemorrhagic stroke patients ($P < .001$).

3.3. The expression levels of lncRNA XIST, miR-340-5p, and PFKFB3 mRNA

Compared to healthy controls, lncRNA XIST expression level was significantly higher in both stroke groups. The highest levels were observed in the hemorrhagic stroke followed by ischemic stroke patients (3.632 ± 0.511 and 1.587 ± 0.092 respectively). Also, PFKFB3 mRNA expression was significantly higher in both stroke groups compared to control group; the hemorrhagic stroke patients showed higher values than those ischemic stroke (3.028 ± 0.372 and 1.554 ± 0.376 respectively). On the other hand, a significant reduction of miR-340-5p expression was observed in stroke patients with the lowest levels observed in the hemorrhagic stroke patients (0.423 ± 0.054 and 0.767 ± 0.052) (Fig. 1) (all $P < .001$).

3.4. Correlation analysis of the studied biochemical and molecular markers with NIHSS scores

On performing Pearson correlation analysis to correlate the studied biochemical and molecular markers with NIHSS scores in stroke patients; significant positive correlation was revealed between NIHSS scores and each of lncRNA XIST and PFKFB3 mRNA expression levels, and the serum levels of each of PFKFB3, VEGF, and HIF-1α, as well. On the other hand, miR-340-5p expression was correlated in the reverse manner (Table 3).

3.5. Multiple linear regression analysis for predicting stroke severity

Multiple linear regression analysis was run using NIHSS score as dependent variable, and our studied parameters as independent variables, it was observed that PFKFB3 mRNA expression (β = 0.7, $P = .003$), was the independent predictor of acute stroke severity (Table 4).

Table 1

Demographic and clinical data of the studied groups.

Variable	Group 1 Control group (N = 40)	Group 2 Ischemic stroke patients (N = 40)	Group 3 Hemorrhagic stroke patients (N = 40)	One-way ANOVA/ Chi²/*t*-test	
				F/χ^2/*t*	*P* value
Gender					
Male	22 (55%)	20 (50%)	26 (65%)	1.9	.387
Female	18 (45%)	20 (50%)	14 (35%)		
	52.13 ± 4.58	53.05 ± 4.91	53.25 ± 5.79		
Age (yr)	–	13.43± 3.11	15.381± 2.92‡	0.549	.579
NIHSS					
Medical history	0 (0%)	7 (17.5%)†	16 (40%)†,‡	–2.898	.005*
Diabetes mellitus, n (%)	0 (0%)	19 (47.5%)†	23 (57.5%)†	20.762	<.001*
Hypertension, n (%)	0 (0%)	13 (32.5%)†	2 (5%)‡	33.19	<.001*
Ischemic heart disease, n (%)	6 (15%)	10 (25%)	7 (17.5%)	22.4	<.001*
Smoking, n (%)	105.15 ± 11.12	120.90 ± 23.35†	119.85 ± 22.29†	1.398	.497
RBG (mg/dL)	162.80 ± 18.49	182.05 ± 27.1†	178.20 ± 21.09†	7.98	.001*
TC (mg/dL)	101.75 ± 9.38	161.30 ± 22.75†	124.55 ± 25.93†,‡	8.18	<.001*
TG (mg/dL)	51.20 ± 4.53	51.05 ± 5.34	48.98 ± 7.25	84.78	<.001*
HDL-c (mg/dL)	91.25 ± 19.86	104.54± 27.5†	102.79 ±20.32	1.83	.165
LDL-c (mg/dL)	0.97 ± 0.09	1.23 ± 0.15†	1.29 ± 0.2†	4	.021*
Creatinine (mg/dL)	11.94 ± 0.23	17.39 ± 0.68†	16.36 ± 2.6†,‡	51.37	<.001*
PT (seconds)	0.98 ± 0.03	1.51 ± 0.12†	1.52 ± 0.12†	138.52	<.001*
INR				484.17	<.001*

Categorical data are presented as numbers (n) and percentage (%); continuous variables are presented as mean ± standard deviation; statistical study was achieved using 1-way ANOVA with post hoc Tukey test for multiple comparisons, chi-square, or *t*-tests.

Abbreviations: HDL-C = high-density lipoprotein–cholesterol, INR = International normalized ratio, LDL-C = low-density lipoprotein–cholesterol, NIHSS = National Institute of Health Stroke Scale, PT = prothrombin time, RBG = random blood glucose, TC = total cholesterol, TG = triacylglycerol.

*Indicates statistical significance.

†Significant difference versus control group (*P* < .05).

‡Significant difference versus ischemic stroke group (*P* < .05).

Table 2

Comparison between HIF-1α, VEGF, PFKFB3 serum levels among the studied groups.

Variable	Group 1 Control group (N = 40)	Group 2 Ischemic stroke patients (N = 40)	Group 3 Hemorrhagic stroke patients (N = 40)	One-way ANOVA	
				F	*P* value
Serum HIF-1α (ng/mL)	0.76 ± 0.04	1.14 ± 0.17†	1.35 ± 0.19†,‡	157.1	<.001*
Serum VEGF (pg/mL)	176.43 ± 31.85	271.39 ± 15.94†	294.05 ± 22.36†,‡	264.33	<.001*
Serum PFKFB3 (ng/mL)	0.52 ± 0.09	2.09 ± 0.17†	2.92 ± 0.24†,‡	1841.6	<.001*

Data is presented as mean ± standard deviation, statistical study was achieved using 1-way ANOVA with post hoc Tukey test.

Abbreviations: HIF-1α = hypoxia inducible factor 1-alpha, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, VEGF = vascular endothelial growth factor.

*Indicates statistical significance.

†Significant difference versus control group (*P* < .05).

‡Significant difference versus ischemic stroke group (*P* < .05).

3.6. Diagnostic performance of the studied markers

ROC curve analysis was applied for lncRNA XIST, miR-340-5p, and PFKFB3 mRNA expression as diagnostic biomarkers of early stroke (Fig. 2A). The optimal cutoff point for lncRNA XIST expression was 1.37, area under the curve (AUC) of 0.996, with sensitivity 96.5% and specificity 97.5%. While, for miR-340-5p expression, the optimal cutoff point was 0.78, AUC of 0.991, with sensitivity 95% and specificity 95%. For PFKFB3 mRNA expression, the optimal cutoff point was 1.34, AUC of 0.981, with sensitivity 87.8% and specificity 95%. Moreover, the potential diagnostic value of these biomarkers for discriminating ischemic stroke patients from hemorrhagic ones was investigated. The optimal cutoff point for lncRNA XIST expression was 2.02, AUC of 0.99, with sensitivity 95% and specificity 95%, while in miR-340-5p expression, the optimal cutoff point was 0.63, AUC of 0.979, with sensitivity 97.5% and specificity 92.5%. For PFKFB3 mRNA expression, the optimal cutoff point was 2.21, AUC of 0.980, with sensitivity 95% and specificity 92.5% (Fig. 2B).

4. Discussion

Revascularization procedures are time-sensitive, and their effects remain limited up to 24 hours for endovascular thrombectomy and to 4.5 hours for intravenous thrombolysis; therefore, early detection of acute ischemic stroke and its differentiation from hemorrhagic type is critical. Thus, a rapid diagnostic test based on blood biomarkers in conjunction with conventional diagnosis methods, including cranial imaging, should be ensured. These biomarkers could allow extensive clinical phenotyping, improve diagnostic precision, predict clinical outcomes, select patients for clinical trials, monitor disease progression, and identify new therapeutic targets. In stroke, the number of candidate biomarkers is persistently increasing, however, no marker can simultaneously enjoy appropriate specificity, sensitivity, promptness, accuracy, and cost effectiveness in routine controlling.[26]

Interestingly, the current study is the first we are aware of to unravel the usefulness of peripheral blood lncRNA XIST, miR-340-5p, and PFKFB3 mRNA expression levels in the diagnosis of acute ischemic and hemorrhagic stroke patients and their correlation to disease severity.

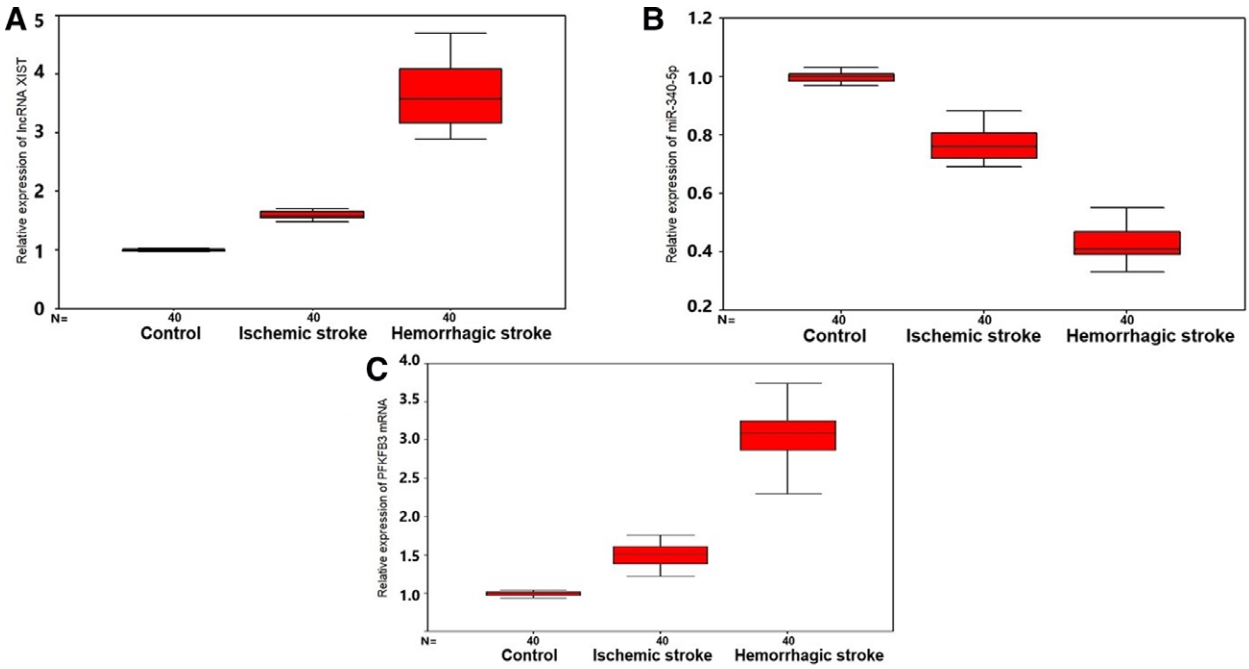

Figure 1. The expression levels of (A) lncRNA XIST, (B) miR-340-5p, (C) PFKFB3 mRNA in the studied groups. The statistical study was achieved using 1-way ANOVA with post hoc Tukey test; [a] Significant difference versus control group ($P < .001$); [b] Significant difference versus ischemic stroke group ($P < .001$). GAPDH = Glyceraldehyde-3-phosphate dehydrogenase, LncRNA XIST = long noncoding RNA XIST, miR-340-5p = microRNA-340-5, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3.

Table 3

Correlation matrix between the studied biochemical and molecular markers and NIHSS scores.

Variable		NIHSS	Serum HIF-1α	Serum VEGF	Serum PFKFB3	PFKFB3 mRNA expression	miR-340-5p expression
Serum HIF-1α	*r*	0.290					
	P	.009*					
Serum VEGF	*r*	0.224	0.320				
	P	.046*	.004*				
Serum PFKFB3	*r*	0.300	0.473	0.455			
	P	.007*	<.001*	<.001*			
PFKFB3 mRNA expression	*r*	0.457	0.537	0.485	0.819		
	P	<.001*	<.001*	<.001*	<.001*		
miR-340-5p expression	*r*	–0.327	–0.450	–0.471	–0.828	–0.860	
	P	.003*	<.001*	<.001*	<.001*	<.001*	
LncRNA XIST expression	*r*	0.333	0.468	0.472	0.856	0.831	–0.920
	P	.003*	<.001*	<.001*	<.001*	<.001*	<.001*

r, Pearson correlation coefficient.

Abbreviations: HIF-1α = hypoxia inducible factor 1-alpha, LncRNA XIST = long noncoding RNA X-inactive specific transcript, miR-340-5p = microRNA-340-5, NIHSS = National Institute of Health Stroke Scale, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, VEGF = vascular endothelial growth factor.

*Indicates statistical significance ($P < .05$).

LncRNAs have vastly been described as key regulators in multiple physiopathology processes.[5] Accordingly, evaluation of their expression levels could be of significance to elucidate the mechanism underlying complex diseases including stroke. Recently, a growing body of evidence has reported the functional value of lncRNA XIST in the pathophysiology of cerebrovascular disorders, but few studies have focused on its clinical value.[10,11]

Our results revealed that lncRNA XIST expression was distinctly increased in peripheral blood from stroke patients in comparison to control subjects, where it was remarkably higher in hemorrhagic than ischemic stroke patients. Concordant with our findings, lncRNA XIST was highly expressed in ischemic brain tissue and involved in neuronal damage and neurological dysfunction, while its depletion attenuated ischemia/reperfusion (I/R)-induced neurological deficits, inflammatory response, and apoptosis as reported by Wang Y et al (2021), Wang J et al, (2021), and Zhang M, et al (2021).[20–22] Decreased lncRNA XIST expression was also reported to restrain oxidative stress and apoptosis in rat hippocampal neurons.[27] Furthermore, it was revealed that upregulated lncRNA XIST had a vital role in hypoxia-induced angiogenesis in human brain microvascular endothelial cells and regulating endothelial cell damage.[28] These molecular mechanisms, in combination with our results support the pathological roles of lncRNA XIST in acute cerebrovascular stroke.

LncRNAs serve as molecular sponges for specific miRNAs to regulate their expression and downstream gene expression. LncRNA XIST has been elucidated to sponge miR-340-5p.[13,14] In the present study, we showed that compared to the control

Table 4

Multiple linear regression analysis for potential predictors of stroke severity.

	Unstandardized coefficients		Standardized coefficients		
Variable	**B**	**Std. Error**	**Beta**	***t***	***P*-value**
LncRNA XIST expression	0.387	0.838	0.134	0.462	.645
miR-340-5p expression	4.632	5.105	0.265	0.907	.367
PFKFB3 mRNA expression	2.663	0.860	0.700	3.097	.003*
Serum PFKFB3	−1.394	1.440	−0.207	−0.968	.336
Serum VEGF	0.003	0.017	0.021	0.174	.862
Serum HIF-1α	0.936	1.839	0.062	0.509	.612
Dependent Variable: NIHSS					

Abbreviations: HIF-1α = hypoxia inducible factor 1-alpha, LncRNA XIST = long noncoding RNA X-inactive specific transcript, miR-340-5p = microRNA-340-5, NIHSS = National Institute of Health Stroke Scale, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, VEGF = vascular endothelial growth factor.

P < .05 is significant.

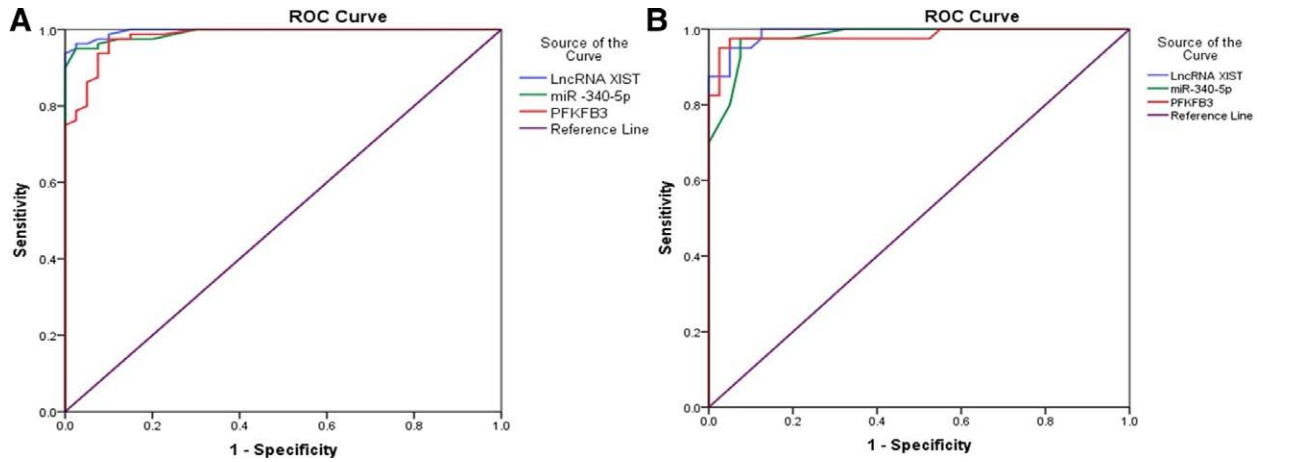

Figure 2. Receiver operating characteristic (ROC) curve of lncRNA XIST, miR-340-5p and PFKFB3 gene expression for (A) early detection of stroke, (B) discriminating ischemic and hemorrhagic stroke. LncRNA XIST = long noncoding RNA XIST, miR-340-5p = microRNA-340-5p, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3.

group, miR-340-5p expression was significantly reduced in both stroke groups, and it was lower in hemorrhagic stroke than ischemic stroke. Current results concur with prior studies reporting low expression of miR-340-5p in acute ischemic and hemorrhagic stroke.[23,24] Recent studies also indicated the protective role of miR-340-5p in cellular and animal models of neuroinflammation.[29,30]

As miRNAs modulate the expression of downstream target genes, the present study proceeded to investigate miR-340-5p downstream signaling molecules, showing significant increase of both HIF-1α and VEGF levels in all stroke patients compared to control and in hemorrhagic more than ischemic stroke patients. This was in accordance with earlier report.[31]

Although it seems to be the first such demonstration, it comes in line with an earlier study validating that miR-340-5p is an upstream regulator of Sirt3,[32] which was demonstrated to regulate HIF-1α/VEGF signaling and exert protective roles *in vivo* and *in vitro* against stroke-induced damage, maintaining blood brain barrier (BBB) integrity and attenuating neuroinflammation.[33] These findings can support the protective role miR-340-5p in hypoxic conditions as stroke.

Under hypoxic conditions, HIF-1α is activated, mediating various pathological processes by regulation of genes involved in oxidative stress, inflammation, apoptosis, autophagy, and energy metabolism. One of HIF-1α target genes is VEGF, which increases substantially during severe and sustained hypoxia leading to BBB disruption.[34] On the other hand, inhibition of HIF-1α in the early acute phase of stroke can significantly downregulate VEGF and improve neuronal survival and sensorimotor function.[35]

PFKFB3 is another downstream target of HIF-1α, it catalyzes 1 of the rate-limiting checkpoints of glycolytic flux. HIF-1α/PFKFB3 signaling has been implicated in the control of pathological angiogenesis,[36] cognitive dysfunction and neuroinflammation,[37] as well as neuronal damage in epilepsy.[38]

The current results showed significant increase of PFKFB3 mRNA expression and its serum level in stroke patients compared to control group, with significant higher levels in hemorrhagic group versus ischemic group. To our knowledge, this is the first study evaluating PFKFB3 transcript in stroke patients' peripheral blood and its serum level. Our findings are supported by previous studies reporting hypoxia-enhanced glycolysis being involved in microglia-mediated inflammatory injury during ischemic and hemorrhagic stroke.[39,40]

Consistently, Li et al reported that during cerebral I/R injury, PFKFB3 expression was upregulated in cortical neurons directing neuronal glucose metabolism to aerobic glycolysis, leading to excessive reactive oxygen species production, mitochondrial dysfunction, and eventually neuronal apoptosis.[41] Furthermore, Yan et al demonstrated that pharmacological inhibition of PFKFB3 enhances resolution of inflammation and improves ischemic brain injury.[25] Accordingly, PFKFB3 could hold a therapeutic strategy to reprogram metabolic profile and improve stroke patients' outcomes.

Of note, lncRNA XIST expression showed strong positive correlation with both PFKFB3 mRNA expression and serum level of PFKFB3, while showed low to moderate positive correlation with each of serum levels of VEGF and HIF-1α, as well as NIHSS score. On the other hand, miR-340-5p expression strongly correlated in the reverse manner. Additionally,

multiple linear regression analysis using NIHSS score as dependent variable, showed that PFKFB3 mRNA expression was independent predictor of acute stroke severity. Indeed, ROC curve analysis revealed that lncRNA XIST, miR-340-5p, and PFKFB3 mRNA expression were useful biomarkers for early stroke detection and discriminating ischemic from hemorrhagic stroke.

Taken together, we could postulate that hypoxia following stroke upregulates lncRNA XIST which sponges miR-340-5p causing upregulation of the HIF1-α/PFKFB3 signaling pathway, resulting in metabolic reprogramming that may have a close relationship with disease severity. Therefore, this study can raise the possibility that dysregulation of lncRNA XIST/miR-340-5p and HIF1α/PFKFB3 pathways might be underlying mediators of stroke pathogenesis.

5. Conclusion

The present study illustrates the upregulation of lncRNA XIST and PFKFB3 and the downregulation of miR-340-5p in stroke patients, recommending collaboration between them, in addition to their role in differentiating stroke type. The study may also propose lncRNA XIST/miR-340-5p and HIF1α/PFKFB3 pathways as probable pathogenesis and therapeutic targets for stroke. Despite, the current work is the first to focus on the association between lncRNA XIST, miR-340-5p and PFKFB3 expression in stroke patients, however we acknowledge that these results need further validation across larger more diverse populations to elucidate the reproducibility and applicability of the drawn findings. Also, using different molecular techniques are needed to clarify the precise molecular interacting mechanisms by which they are involved in the pathogenesis of stroke and to explore their potential role as targets for stroke treatment.

Author contributions

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