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Circulating lncRNAs HIF1A-AS2 and LINLK-A: Role and Relation to Hypoxia-Inducible Factor-1α in Cerebral Stroke Patients

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Abstract

Long noncoding RNAs (lncRNAs) have been recently recognized as key players of gene expression in cerebral pathogenesis. Thus, their potential use in stroke diagnosis, prognosis and therapy is actively pursued. Due to the complexity of the disease, identifying stroke- specific lncRNAs remains a challenge. This study investigated the expression of lncRNAs; HIF1A-AS2 and LINK-A and their target gene Hypoxia-inducible factor-1 (HIF-1) in Egyptian stroke patients. It also aimed to determine the molecular mechanism implicated in the disease. A total of 75 stroke patients were divided into three clinical subgroups, beside 25 healthy controls of matched age and sex. Remarkable upregulation of lncRNA HIF1A-AS2 and HIF1-α along with a downregulation of lncRNA LINK-A were noticed in all stroke groups relative to controls. Serum levels of phosphatidylinositol 3- kinase (PI3K), phosphorylated-Akt (p-Akt), vascular endothelial growth factor (VEGF) and angiopoietin-1 (ANG1) as well as their receptors, malondialdehyde (MDA) and total antioxidant capacity (TAC) were significantly increased, whereas brain-derived neurotrophic factor (BDNF) levels were significantly decreased particularly in hemorrhagic stroke versus ischemic groups. Eventually, these findings support the role of lncRNAs; HIF1A-AS2 and LINK-A as well as HIF1-α in activation of angiogenesis, neovascularization, and better prognosis of stroke, especially the hemorrhagic type.

Introduction:

Cerebral stroke is a major health problem affecting a large population in both developed and developing countries [1]. Without rapid successful intervention, stroke can cause sudden cerebral death or severe prolonged disability [1]. Two main types of stroke are identified: ischemic stroke (thrombotic and embolic) and hemorrhagic stroke (cerebral and subarachnoid). Although hemorrhagic stroke is much more fatal, prevalence and incidence rates of thrombotic showed the highest ones, followed by cerebral hemorrhage, then embolic and lastly subarachnoid hemorrhage [2].

Among the difficulties for timely treatment and management of stroke are the complexity of the disease resulting from its multiple underlying risk factors, lack of sensitive and specific biomarkers for easily diagnosis and prognosis as well as potential therapy. Therefore, the need for more reliable biomarkers is highly desired. It is known that apparent changes in the expression of multiple genes in cerebrum are a major cause as well as useful predictors of the pathogenesis in cerebral stroke. Identification of such genes, particularly those that are highly specific and sensitive to stroke may be the key step towards reliable prediction of stroke. Hence, enhanced understanding of stroke pathogenesis would aid in the early detection and optimal disease management.

In stroke, large sequence of biochemical and molecular pathways, causing neuron cell death, were previously linked to the imbalance between oxygen supply and demand in the cerebral tissue. Hypoxic conditions, associated with cerebrovascular stroke, are a strong challenge for oxygen-dependent mammalian cells requiring adequate cellular response to fine-tune proliferative and metabolic processes. The main transcriptional regulator of cellular response to hypoxia is the hypoxia-inducible factor (HIF) [3].

HIF is a heterodimeric transcription factor consisting of an inducible HIF-1α subunit and a constitutive HIF-1β subunit. Under hypoxic conditions, the HIF-1α subunit accumulates, and forms HIF-1α-HIF-1β heterodimer that undergoes posttranslational alterations and promotes transactivation [4]. This will enable binding to hypoxia response elements (HRE) in the promoter regions of target genes that enhance angiogenesis, cellular supply with oxygen, erythropoiesis, vascular tone maintenance, energy providing substrates and cell survival [5]. HIF-1α could thus protect neurons against oxidative stress-induced apoptosis and focal cerebral ischemia [6]. These cellular adaptive response is regulated by growth factor-dependent signaling pathways, including phosphoinositide 3-kinase (PI 3-kinase)/AKT cascades [7].

Recent studies have assured that the PI3K/AKT signaling pathway can regulate multiple pro-angiogenic factors, including mammalian target of rapamycin (mTOR), vascular endothelial growth factor (VEGF), HIF-1α, nitric oxide (NO), and Angiopoietin-1 (ANG1) [8]. Activation of these exogenous angiogenic factors can motivate angiogenesis in the peri-ischemic tissue, thereby composing a new compensatory collateral circulation to enhance the blood supply to ischemic/peri-ischemic tissue [9].

Long noncoding RNAs (lncRNAs) are a class of non-protein coding RNAs, that were reported to play an important role in the modulation of the protein-coding genes expression at all regulation levels, including transcriptional and posttranscriptional control, translational, post translation and epigenetic regulation [10, 11]. It has been found that lncRNAs are upregulated upon hypoxia, acting directly or indirectly as stimulators or inhibitors of the HIF-pathway [12]. Lorenzen and Thum. [13] have also demonstrated that lncRNAs might be involved in the regulation of angiogenesis as well as the pathophysiologic processes of ischemic stroke since their expression profiles were altered in the peripheral blood of stroke patients. Therefore, understanding the role of lncRNAs in stroke pathogenesis is of particular importance to boost their use as predicting biomarkers of stroke.

LncRNA HIF1A antisense RNA 2 (HIF1A-AS2) was found to regulate HIF-1α mRNA as the putative HIF-1α protein binding sites-HREs were proposed to be located in the HIF1A-AS2 promoter region [14]. Furthermore, Li et al., [15] reported that lncRNA HIF1A-AS2 could promote angiogenesis in human umbilical vein endothelial cells (HUVECs) during hypoxia via facilitating the up-regulation of HIF-1 α. However, its regulatory mechanisms in stroke have not been well investigated.

In the meantime, another lncRNA was addressed here, which is long intergenic non-coding RNA for kinase activation (LINK-A). In 2016, LINK-A was identified as cytoplasmic and highly prognostic lncRNA in triple-negative breast cancers which is responsible for HIF1-α stabilization and activation of HIF1-α transcriptional programs under normoxic conditions, promoting breast cancer glycolysis reprogramming and tumorigenesis [16]. On the other side, knockdown of LINK-A in both MDA-MB231 and MDA-MB-468 cells eliminated its dependent kinases phosphorylation and HIF1-α stabilization. In contrary, under hypoxia LINK-A knockdown exhibited minimal effects on hypoxia-dependent HIF1-α stabilization [16]. Additionally, the existence of an association between LINKA and HIF1α in osteosarcoma has been documented by Zhao et al., [17] who indicated that LINKA overexpression had inhibitory effects on HIF1α expression, whereas HIF1α had no effect on LINKA.

The possibility that these lncRNAs are dynamically deregulated during hypoxia in stroke stimulate our interest to measure the expression level of these lncRNAs in sera of patients with ischemic and hemorrhagic stroke. Being exceptionally stable in the bloodstream and readily detectable in human subjects with tissue injury, the potential use of these lncRNAs as non-invasive and rapid diagnostic and prognostic tools for stroke will be promising.

In this context, the present study aimed to investigate the regulatory role of lncRNAs; HIF1A-AS2 and LINK-A in relation to HIF1-α in the development of stroke in Egyptian patients. The study was also directed to identify the molecular mechanisms implicated in acute cerebral stoke.

Subjects And Methods:

1- Participants:

This study included 100 participants, 25 healthy controls and 75 stroke patients recruited from stroke Clinic, Neurology Department, Kasr Al-Ainy Hospital, Cairo University from March 2019 to March 2020. A confirmed clinical diagnosis and characterization of stroke subtypes were done by a neurologist based on either computed tomography (CT) or magnetic resonance imaging (MRI) of the brain. Eligible patients were defined as those who have diagnosed with acute stroke according to neurological examination and radiological imaging, including a sudden onset of focal neurological deficit for more than 24 h with corresponding infarction on brain imaging. Stroke patients were thus stratified into 3 subtypes, each of 25; i- thrombotic patients (19 male/6 female) with age range (39–86), ii- embolic patients (16 male/9 female) with age range (45–85) and iii- hemorrhagic patients (16 male/ 9 female) with age range (37–87). The risk factors; diabetes, hypertension, smoking, ischemic heart disease and hyperlipidemia were characterized based on medical history and routine laboratory tests [11]. The exclusion criteria included any current or recent transient ischemic attack, cerebral trauma, cerebrovascular malformations, coagulation disorders, autoimmune diseases, tumors, and chronic infection diseases. Renal and liver diseases, homeopathy, and occlusive arterial disease or phlebothrombosis of limbs were also excluded.

As regards the controls, 25 healthy individuals (17 male/ 8 female) with age range (43–72) volunteered to participate in this study. All participants gave written informed consent. The study protocol was approved by the Research and Ethics committee for Experimental and Clinical studies at Faculty of Pharmacy, Cairo University, Cairo, Egypt and the study was conducted according to the guidelines of the declaration of Helsinki, revised in 2008.

2- Sample Collection and Biochemical Measurements:

Five ml venous blood were collected from all participants (from stroke patients within the first 24 h from the onset of symptoms) using serum collection tubes. The separated sera were aliquoted and stored at −80°C for the assessment of lncRNAs, mRNA HIF1-α, vascular endothelial growth factor (VEGF), angiopoietin-1 (ANGPT1), brain-derived neurotrophic factor (BDNF), oxidative stress biomarkers and the protein expression of phosphatidylinositol 3- kinase (PI3K), phosphorylated-Akt (p-Akt), VEGFR2 and

angiopoietin-1 receptor (TIE2) receptors. An aliquot of the serum was used to assess the routine workup, full lipid profile and prothrombin time.

Serum lncRNAs and mRNA assay using real time polymerase chain reaction (qRT-PCR)

Total RNAs Isolation and qRT-PCR:

Total RNA was extracted from 200 µL serum by the miRNeasy Mini Kit (Qiagen, Hilden, German) using QIAZOL lysis reagent according to the manufacturer's instructions. The extracted RNA was dissolved in 50 µL RNase-free water and stored at − 80°C until analysis. The quality of RNA was determined using nanodrop UV-Visible Spectrophotometer (Thermo Scientific, United States). The purity range for the samples was (1.8-2-0) at wavelength 260/280 [18], whereas the RNA yield range was (700–1400) nanogram.

Reverse transcription was done using RT2 first strand Kit (Qiagen, Hilden, Germany). 8 µL total RNA template were reverse transcribed in a final reaction mix volume of 20 µL. For synthesis of cDNA, the RT reaction was incubated for 60 min at 37°C, and for 5 min at 95°C. The cDNA produced were stored at − 20°C till analysis.

Relative expression levels of lncRNAs; HIF1A-AS2 and LINK-A along with the gene expression of HIF-1α were evaluated using the RT2 SYBR Green Master Mix kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The housekeeping gene, GAPDH, was selected as the internal control. Briefly, for the analysis of lncRNAs; HIF1A-AS2 and LINK-A, 2 µL cDNA product was used as a template in 25 µL total reaction volume containing 12.5 µL RT2 SYBR Green PCR Master Mix, 9.5 µL nuclease-free water, and 1 µL RT2 LncRNA PCR primer assay. Readily made primers by Qiagen were used for amplification. The primer sequences were provided in table (1). qRT-PCR was performed with a Qiagen Rotor Gene Q6 Plex Real-Time PCR system (Qiagen, Hilden, Germany), with a PCR initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

For the assessment of mRNA HIF1-α relative expression, qRT-PCR was performed in 25 µL reaction mixture prepared by mixing 12.5 µL master mix, 2.5 µL primer assay, 5 µL cDNA, and 5 µL RNAase-free water. The reaction was performed with a PCR initial activation at 95°C for 15 min followed by 40 cycles at 95°C for 15 s, 55°C for 30 s and 72°C for 30 s.

The data were examined with Rotor Gene Q software with the automatic threshold cycle (Ct) setting. The relative expression for each lncRNA as well as mRNA HIF1-α after normalization to GAPDH was calculated using the $2^{-\Delta\Delta ct}$ method.

Serum MDA and TAC Levels:

Serum Malondialdehyde (MDA) and serum Total Antioxidant Capacity (TAC) concentrations were assayed using colorimetric kit (Bio Diagnostic, Cairo, Egypt). MDA and TAC levels were expressed as nmol/ml and mM/L, respectively.

Serum VEGF, ANG-1 and BDNF Levels:

Serum concentration of VEGF, ANG-1 and BDNF were assessed using ELISA Kit (My Bio Source, California, USA) (Catalog Number. MBS355343), (Catalog Number. MBS700602) and (Catalog Number. MBS905339), respectively according to the manufacture instructions and expressed as pg/ml for VEGF and ng/ml for both ANG-1 and BDNF.

Protein expression of PI3K, AKT, VEGFR2 and TIE:

Cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad, California, USA). The membranes were blocked with 5% nonfat dry milk for 1 h and probed with specific primary antibodies of anti-PI3K (Thermo Fisher Scientific, Massachusetts, USA), anti-pAkt (Thermo Fisher Scientific, Massachusetts, USA), anti-TIE (Thermo Fisher Scientific, Massachusetts, USA), and anti-VEGFR2 (Thermo Fisher Scientific, Massachusetts, USA), followed by incubation with the appropriate peroxidase conjugated secondary antibodies (Dianova, Hamburg, Germany) for 1 h. Finally, chemiluminescence (Bio-Rad, California, USA) was utilized to visualize the protein banding and β-actin was used as an internal control.

Statistical analysis:

The results were presented as mean ± standard error of mean (M ± SEM). Both parametric and nonparametric statistical methods were used to give full study of stroke different types. Power analysis was conducted for a one-way fixed effect analysis of variance (ANOVA) to compare between different groups and post hoc Tukey was utilized to compare individual groups. Nonparametric receiver operating characteristic (ROC) curves were created between ischemic stroke groups and hemorrhagic stroke group in which the value for sensitivity is plotted against 1-specificity. A prognostic test (positive vs. negative) was conducted for ischemic and hemorrhagic groups using a cutoff threshold for HIF1-α, HIF1A-AS2 and LINK-A. The positivity rates were compared by chi-square test. The overall accuracy of a molecular marker to predict stroke different types is defined as the average of the sensitivity and the specificity.

Simple linear regression analysis was applied using Pearson $\chi 2$ test to study the correlation between serum levels of mRNA HIF1-α, lncRNAs; HIF1A-AS2 and LINK-A with each other and with demographic, clinical data, VEGF, ANG1, BDNF, MDA and TAC. Prognostic binary logistic regression analysis was performed between ischemic and hemorrhagic patients using the measured parameters to assess their potential use as prognostic tools in a univariate fashion. P-values < 0.05 were considered statistically significant. Odds ratio and 95% confidence interval (CI) were calculated. All statistical analysis was performed by using windows-based SPSS statistical software (SPSS version 20.0, SPSS Inc., Chicago, IL) and GraphPad Prism 7.0 (GraphPad Software, CA, USA).

Results:

Demographic and clinical characteristics of the study participants:

Table (2) provides demographic and clinical characteristics of the study participants. Of the 75 stroke patients; 29 were diabetic, 44 were hypertensive, 26 were smokers and 14 suffered from ischemic heart disease. All stroke groups exhibited significantly higher serum levels of cholesterol, LDL, VLDL and risk ratio, without any significant differences in triglycerides, HDL-cholesterol and prothrombin time compared to healthy controls.

Serum expression levels of mRNA HIF1-α and LncRNAs in healthy controls and stroke groups:

Compared to healthy controls, the expression levels of mRNA HIF1-α and lncRNA HIF1A-AS2 were markedly upregulated, while those of lncRNA LINK-A were substantially downregulated in all stroke groups at p =0.000. Notably, mRNA HIF1-α and lncRNA HIF1A-AS2 reached their highest level in hemorrhagic group, whereas thrombotic group displayed the least increase as compared to other stroke groups. On the other hand, lncRNA LINK-A was significantly low in hemorrhagic group compared to thrombotic and embolic groups, without any significance difference in its expression level between both ischemic groups (Fig. 1).

Serum levels of MDA and TAC in healthy controls and stroke groups:

In all stroke patients, the serum levels of oxidative stress biomarkers were significantly high in comparison with healthy controls. MDA levels were markedly high in hemorrhagic group compared with thrombotic and embolic groups, without any significant difference between ischemic groups. On the other side, increased serum levels of TAC did not show significant difference among the three studied groups of stroke patients (Fig. 2).

Serum levels of VEGF, ANG1 and BDNF in healthy controls and stroke groups:

In all stroke groups, serum levels of VEGF and ANG-1 were significantly high, whereas BDNF levels were significantly low as compared to healthy controls (Fig. 2). Obviously, serum VEGF was markedly increased in hemorrhagic stroke compared to thrombotic and embolic groups, without any significant difference between the last two groups. Similarly, the hemorrhagic group showed the highest ANG-1 serum levels while the embolic group showed the lowest level. However, there was no significant difference in BDNF serum level between stroke groups.

Serum protein expression levels of PI3K, P-Akt, VEGFR2 and TIE2 in healthy controls and stroke groups:

Results in figure (3) illustrated that the serum protein levels of PI3K, P-Akt, VEGFR2 and TIE2 were significantly higher in all groups of stroke patients versus healthy controls, without any significant difference between the stroke groups versus each other.

Correlation analysis of different serum molecular markers and clinical data in all stroke groups:

To evaluate the usefulness of circulating mRNA HIF1-α and lncRNAs; HIF1A-AS2 and LINK-A as stroke biomarkers, we tested whether their levels were associated with stroke risk factors and biochemical markers.

Pearson's correlation analysis revealed significant positive correlation of HIF1-α and HIF1A-AS2 with each other and with diabetes mellitus, hypertension, TAGs, serum-cholesterol, LDL, VLDL, risk ratio, prothrombin time, MDA, TAC, VEGF, ANG1, and significant negative correlation with lncRNA LINK-A and BDNF. On the other side lncRNA LINK-A was correlated in opposite manner (Table 3).

ROC curve and positivity rate:

ROC curve analysis was applied to test the possible use of mRNA HIF1-α, lncRNAs; HIF1A-AS2 and LINK-A in predicting hemorrhagic stroke patients from ischemic ones as presented in figure (5) and tables (4,5). All biomarkers were efficient in predicting hemorrhagic from ischemic patients. For HIF1-α, the optimal cut-off value was 7.17-fold change giving 80% sensitivity and 84% specificity and area under the curve (AUC) equivalent to 0.83, whereas for HIF1A-AS2 and LINK-A the optimal cut-off values were 7.83- and 0.28-fold change giving 80% and 92% sensitivity and 82% and 94% specificity, with AUC equivalent to 0.867 and 0.914, respectively. Regarding the positivity rates for HIF1-α, lncRNA HIF1A-AS2 and lncRNA LINK-A, they were 80, 80 and 92 %, respectively in the serum of the hemorrhagic stroke patients compared to 16, 18 and 6 %, respectively in ischemic stroke patients (Fig. 4).

Univariate logistic regression analysis for predicting hemorrhagic stroke:

To predict the risk of hemorrhagic stroke, univariate logistic regression analysis was conducted. Data indicated that HIF1-α, HIF1A-AS2, LINK-A, VEGF and ANG1 were significant predictors for hemorrhagic stroke with odds ratios; 1.984, 2.201, 2.537, 1.016 and 1.243, respectively. Remarkably, LINK-A showed the highest odds ratio (2.537) (Table 5).

Discussion:

Hypoxia occurs commonly after stroke and is usually associated with poor clinical and functional outcomes [19]. Interestingly, this study is the first to investigate the usefulness of serum lncRNAs; HIF1A-AS2 and LINK-A expression in diagnosis and prognosis of ischemic and hemorrhagic stroke patients. Moreover, the study emphasized their relation to HIF1-α with regards to PI3K/AKT, angiogenesis and oxidative stress pathways.

Previously, it has been found that HIF-1α protein level is increased under ischemic and hypoxic conditions. Increased HIF-1α abundance is proposed to be related to inhibited degradation mediated by ubiquitination. However, evidence that HIF-1α abundance is controlled by lncRNA HIF1A-AS2 has been also suggested [20]. In tune, Wang et al., [1] have reported that hypoxia induces the expression of lncRNA HIF1A-AS2 in human umbilical vein endothelial cells (HUVECs). Moreover, Li et al., [15] provided a novel mechanism where lncRNA HIF1A-AS2 facilitates the up-regulation of HIF-1α by serving as a 'sponge' to

miR-153-3p, which lessened the post-transcriptional silencing of HIF-1α. Herein, this finding was confirmed by the co-overexpression of both lncRNA HIF1A-AS2 and mRNA HIF1-α in stroke patients as compared to control. Remarkedly, their expressions were higher in hemorrhagic than ischemic stroke patients.

On the contrary, the expression of lncRNA LINK-A was significantly decreased in all stroke patients in comparison to control and in ischemic than hemorrhagic stroke patients. This can be supported by the role of lncRNA LINK-A in activation of normoxic HIF1-α rather than hypoxic HIF1-α [17]. Thus, we postulated that hypoxia following stroke increased the expression of lncRNA HIF1A-AS2 and reduced that of lncRNA LINK-A, causing the encountered upregulation of HIF1- α expression level in those patients.

Hypoxic conditions were reported to trigger an increase of reactive oxygen species (ROS) and to create a state of oxidative stress [21]. In the current study, stroke patients showed increased accumulation of MDA and TAC associated with an enhanced expression of HIF1- α. Indeed, the observed positive correlations between MDA, TAC, and lncRNA HIF1A-AS2 along with their negative correlations with lncRNA LINK-A pointed to a proposed role of these oxidative stress biomarkers in controlling the expression of the studied lncRNAs and consequently HIF1- α.

Formerly, the role of PI3K/Akt signaling pathway in HIF1- α activation under hypoxic condition was documented by [22]. Zhang et al., [23] have also stated that both p-Akt and HIF-1α protein levels increased in response to hypoxia in human mesenchymal stem cells and that p-Akt expression peaked earlier than HIF-1α. Additionally, lncRNA HIF1A-AS2 was found to activate PI3K/AKT signaling pathway through sponging miR-665 leading to upregulation of IL-6 and activation of PI3K/AKT [24]. In agreement, PI3K and p-AKT protein levels of all stroke patients were significantly upregulated in the present study, advocating the role of lncRNA HIF1A-AS2 in the upregulation of PI3K and P-Akt protein levels and consequently HIF-1α activation in the studied patients.

In this study, VEGF and ANG1 levels as well as the protein levels of their receptors (VEGFR2 and TIE2) were significantly increased in all stroke patients. Moreover, these increments were clearly apparent in hemorrhagic compared to ischemic patients. In fact, angiogenesis plays an important role in the repair of tissues subjected to ischemic insult [25]. Biological signals such as hypoxia, ischemia, and/or blood vessel damage upregulate the expression of proangiogenic growth factors and activate their receptors [26]. Meanwhile, vascular permeability increases in response to VEGF, thereby allowing the extravasation of plasma proteins, forming a primitive scaffold for migrating endothelial cells [27]. Alternatively, ANG1, a natural inhibitor of vascular permeability, exerts antagonistic functions during vessel development to protect against plasma leakage [28]. A positive correlation between lncRNA HIF1A-AS2, HIF1- α, VEGF and ANG1, along with a negative correlation between lncRNA LINK-A and VEGF and ANG1 were observed in this study. Hence, we could hypothesize that in stroke patients, overexpression of lncRNA HIF1A-AS2 and down-expression of lncRNA LINK-A levels might lead to increased HIF1- α accumulation, triggering the upregulation of proangiogenic factors and initiating a cascade of new vessels formation.

BDNF is an important neuroprotective factor for ischemic brain injury in vivo that downregulates the expression of some inflammatory cytokines such as tumor necrosis factor α (TNF-α) [29] and decreases apoptosis [30]. BDNF may also activate many intracellular signaling pathways, as PI3K/Akt, thereby affecting both development and function of the nervous system [31]. Decreased BDNF levels observed in stroke patients could be explained on the basis that in acute stroke, BDNF may pass through the blood brain barrier (BBB) probably due to a stroke related disruption of the BBB. This causes substantial increase in extracellular BDNF in the central nervous system that might be reflected by a decrease in serum BDNF levels [32]. In addition, the present results notified a negative correlation between lncRNA HIF1A-AS2, HIF1- α and BDNF along with a positive correlation between lncRNA LINK-A and BDNF, which verify the protective role of BDNF during hypoxia.

Herein, the positivity rates for HIF1-α, lncRNA HIF1A-AS2 and lncRNA LINK-A were 80, 80 and 92 %, respectively, in the serum of the hemorrhagic stroke patients compared to 16, 18 and 6 %, respectively, in ischemic stroke patients. These outcomes boost the role of these lncRNAs and HIF1-α as prognostic biomarkers for stroke, especially the hemorrhagic type. Additionally, on performing binary logistic regression analysis, HIF1-α, HIF1A-AS2, LINK-A, VEGF and ANG1 were found to be significant predictors of hemorrhagic stroke.

Remarkably, the demographic and clinical characteristics data of patients showed that diabetes, hypertension and hyperlipidemia are the strongest risk factors for all stroke subtypes as confirmed by Pearson correlation coefficient. Additionally, smokers were found to be more prone to thrombotic stroke, whereas ischemic heart disease was strongly correlated with embolic stroke only.

In conclusion, cerebral hypoxia usually occurs after stroke leading to increased ROS production. This triggers the upregulation of lncRNA HIF1A-AS2 and downregulation of lncRNA LINK-A, which activate HIF1-α that in turn enhances the expression of VEGF and ANG1 as well as their receptors.

Eventually, the study supports the use of lncRNAs; HIF1A-AS2 and LINK-A as diagnostic and prognostic tools in all stroke patients, especially those with hemorrhagic stroke.

Declarations

Data Availability:

The authors confirm that the data supporting the findings of this study are available within the article. The raw data are available from Rana K. Zayed (rana.zayed@fue.edu.eg) upon reasonable request.

Author Contributions:

The authors acknowledge the work and support provided by each member in this research. Sample collection, preparation, RT-PCR and Elisa analyses and statistical analysis were performed by Rana K. Zayed and Heba A. Ewida. Paper writing, and revision were done by all the contributing members. The paper was read, revised, and approved by all the authors.

Compliance with ethical standards:

The study protocol was approved by the Research and Ethics committee for Experimental and Clinical studies at Faculty of Pharmacy, Cairo University, Cairo, Egypt and the study was conducted according to the guidelines of the declaration of Helsinki, revised in 2008.

Consent to participate:

All participants gave written informed consent and signed by them. All consents are available upon request.

Consent for publication:

Not applicable

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Conflict of interest and discloser statement:

All authors confirm that there is no conflict of interest

References

1. Wang S-W, Liu Z, Shi Z-S (2018) Non-coding RNA in acute ischemic stroke: mechanisms, biomarkers and therapeutic targets. Cell transplantation 27(12):1763–1777. https://doi.org/10.1177/0963689718806818
2. Kandil M et al (2006) Epidemiology of cerebrovascular stroke and TIA in Upper Egypt (Sohag)–relative frequency of stroke in Assiut University Hospital. Egypt J Neurol Psychiat Neurosurg 43(1):593–602
3. Amalia L et al (2020) Hypoxia-inducible factor-1α in acute ischemic stroke: Neuroprotection for better clinical outcome. Heliyon 6(6):e04286. https://doi.org/10.1016/j.heliyon.2020.e04286
4. Hudson CC et al (2002) Regulation of hypoxia-inducible factor 1α expression and function by the mammalian target of rapamycin. Molecular cellular biology 22(20):7004–7014.

10.1128/MCB.22.20.7004-7014.2002

5. Lee JW et al (2019) Hypoxia signaling in human diseases and therapeutic targets. Experimental molecular medicine 51(6):1–13. https://doi.org/10.1038/s12276-019-0235-1
6. Dong Y et al (2013) Protective effect of HIF-1α against hippocampal apoptosis and cognitive dysfunction in an experimental rat model of subarachnoid hemorrhage. Brain research 1517:114–121. https://doi.org/10.1016/j.brainres.2013.04.024
7. Jiang B-H et al., (2001) Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth and Differentiation-Publication American Association for Cancer Research. 12(7): p. 363–370
8. Karar J, Maity A (2011) PI3K/AKT/mTOR pathway in angiogenesis. Front Mol Neurosci. 4: 51. https://doi.org/10.3389/fnmol.2011.00051
9. Wang K, Lin R-Z, Melero-Martin JM (2019) Bioengineering human vascular networks: trends and directions in endothelial and perivascular cell sources. Cell Mol Life Sci 76(3):421–439. https://doi.org/10.1007/s00018-018-2939-0
10. Klingenberg M et al (2017) Non-coding RNA in hepatocellular carcinoma: Mechanisms, biomarkers and therapeutic targets. Journal of hepatology 67(3):603–618. https://doi.org/10.1016/j.jhep.2017.04.009
11. Zhang Z (2014) The Dual Role of Hypoxia-Inducible Factor-1 in Ischemic Stroke: Neuroprotection and Blood-Brain Barrier Disruption. University of Kansas. http://dissertations.umi.com/ku:13572
12. Choudhry H, Harris AL, McIntyre A (2016) The tumour hypoxia induced non-coding transcriptome. Molecular aspects of medicine 47:35–53. https://doi.org/10.1016/j.mam.2016.01.003
13. Lorenzen JM, Thum T (2016) Long noncoding RNAs in kidney and cardiovascular diseases. Nat Rev Nephrol 12(6):360. 10.1038/nrneph.2016.51
14. Chen D et al (2017) Comparison of HIF1A–AS1 and HIF1A–AS2 in regulating HIF–1α and the osteogenic differentiation of PDLCs under hypoxia. International journal of molecular medicine 40(5):1529–1536
15. Li L et al (2017) lncRNAs HIF1A-AS2 facilitates the up-regulation of HIF-1α by sponging to miR-153-3p, whereby promoting angiogenesis in HUVECs in hypoxia. Biomedicine Pharmacotherapy 96:165–172. https://doi.org/10.1016/j.biopha.2017.09.113
16. Lin A et al (2016) The LINK-A lncRNA activates normoxic HIF1α signalling in triple-negative breast cancer. Nature cell biology 18(2):213–224. 10.1038/ncb3295
17. Zhao B, Liu K, Cai L (2019) LINK–A lncRNA functions in the metastasis of osteosarcoma by upregulating HIF1α. Oncology letters 17(6):5005–5011
18. Fleige S, Pfaffl MW (2006) RNA integrity and the effect on the real-time qRT-PCR performance. Molecular aspects of medicine 27(2–3):126–139. https://doi.org/10.1016/j.mam.2005.12.003
19. Ferdinand P, Roffe C (2016) Hypoxia after stroke: a review of experimental and clinical evidence. Experimental translational stroke medicine 8(1):1–8. 10.1186/s13231-016-0023-0

20. Li LJ et al (2014) miR–376b–5p regulates angiogenesis in cerebral ischemia. Mol Med Rep 10(1):527–535
21. McGarry T et al., (2018) Hypoxia, oxidative stress and inflammation. Free Radical Biology and Medicine. 125: p. 15–24. https://doi.org/10.1016/j.freeradbiomed.2018.03.042
22. Kilic-Eren M, Boylu T, Tabor V (2013) Targeting PI3K/Akt represses Hypoxia inducible factor-1α activation and sensitizes Rhabdomyosarcoma and Ewing's sarcoma cells for apoptosis. Cancer cell international 13(1):1–8
23. Zhang J, Zhang Q (2018) VHL and hypoxia signaling: beyond HIF in cancer. Biomedicines 6(1):35. https://doi.org/10.3390/biomedicines6010035
24. Wu R et al (2018) Long non-coding RNA HIF1A-AS2 facilitates adipose-derived stem cells (ASCs) osteogenic differentiation through miR-665/IL6 axis via PI3K/Akt signaling pathway. Stem Cell Res Ther 9(1):1–13. https://doi.org/10.1186/s13287-018-1082-z
25. Bai W-W et al (2013) Tongxinluo improves cardiac function and ameliorates ventricular remodeling in mice model of myocardial infarction through enhancing angiogenesis. Evidence-based complementary alternative medicine. https://doi.org/10.1155/2013/813247
26. Carmeliet P (2003) Angiogenesis in health and disease. Nature medicine 9(6):653–660
27. Eliceiri BP et al (1999) Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Molecular cell 4(6):915–924. https://doi.org/10.1016/S1097-2765(00)80221-X
28. Thurston G et al (2000) Angiopoietin-1 protects the adult vasculature against plasma leakage. Nature medicine 6(4):460–463
29. Makar TK et al (2009) Brain-derived neurotrophic factor gene delivery in an animal model of multiple sclerosis using bone marrow stem cells as a vehicle. J Neuroimmunol 210(1):40–51. https://doi.org/10.1016/j.jneuroim.2009.02.017
30. Islam O, Loo TX, Heese K (2009) Brain-derived neurotrophic factor (BDNF) has proliferative effects on neural stem cells through the truncated TRK-B receptor, MAP kinase, AKT, and STAT-3 signaling pathways. Current neurovascular research 6(1):42–53. https://doi.org/10.2174/156720209787466028
31. Yao R-Q et al (2012) Quercetin attenuates cell apoptosis in focal cerebral ischemia rat brain via activation of BDNF–TrkB–PI3K/Akt signaling pathway. Neurochem Res 37(12):2777–2786. 10.1007/s11064-012-0871-5
32. Di Lazzaro V et al (2007) BDNF plasma levels in acute stroke. Neurosci Lett 422(2):128–130. https://doi.org/10.1016/j.neulet.2007.06.001

Tables

Table 1: Primer sequences used for real-time PCR.

Gene Sequence	
HIF1A-AS2	**Forward** 5´-TCTGTGGCTCAGTTCCTTTTGT-3´
	Reverse 5´-ATGTAGGAAGTGCCAGAGCC-3´
LINK-A	**Forward** 5´-TTCCCCCATTTTTCCTTTTC-3´
	Reverse 5´-CTCTGGTTGGGTGACTGGTT-3´
HIF1-α	**Forward** 5´-ATGAGCCAGAAGAACTTTTAGG-3´
	Reverse 5´-ACCTCTTTTGGCAAGCATCCTG-3´
GAPDH	**Forward** 5´-GGAGCGAGATCCCTCCAAAAT-3´
	Reverse 5´-GCTGTTGTCATACTTCTCATGGA-3´

Table 2: Demographic and clinical characteristics of studied groups:

Groups	Healthy Control	Stroke Patients			*P* value
Parameters	**N= 25**	**Thrombotic N= 25**	**Embolic N= 25**	**Hemorrhagic N= 25**	
Age (Y) Median (Range)	57 (43 – 72)	57 (39 – 86)	64 (45 – 85)	64 (37 – 87)	0.421
Gender:					
Male n (%)	17 (68%)	19 (76%)	16 (64%)	16 (64%)	0.776
Female n (%)	8 (32%)	6 (24%)	9 (36%)	9 (36%)	
Medical History:					
Diabetes mellitus n (%)	0 (0%)	8 (32%) [a]	8 (32%) [a]	13 (52%) [a]	0.001
Hypertension n (%)	0 (0%)	13 (52%) [a]	15 (60%) [a]	16 (64%) [a]	0.000
Smoking n (%)	8 (32%)	16 (64%)	5 (20%) [b]	5 (20%) [b]	0.002
Ischemic heart disease n (%)	0 (0%)	5 (20%)	7 (28%) [a]	2 (8%)	0.022
Lipid Profile:					
Total cholesterol (mg/dl)	133.08±5.799	180.8±8.939	192.96±8.685	194.92±7.726	0.000
LDL (mg/dl)	80.76±2.167	116.36±7.587	129±9.338	121.64±6.595	0.000
HDL (mg/dl)	48±1.507	44.56±2.478	43.08±1.523	43.8±2.9	0.400
TAGs (mg/dl)	95.8±4.851	118.96±7.708	120.8±7.564	120.68±14.453	0.171
VLDL (mg/dl)	12.24±1.093	32.04±3.482	28.88±2.817	30.2±2.965	0.000
Risk Ratio	2.8±0.127	4.344±0.326	4.644±0.288	4.812±0.278	0.000
Prothrombin Time	12.002±0.113	13.202±0.276	13.068±0.312	13.156±0.232	0.002

N: number, Y: years, LDL: low density lipoprotein, HDL: high density lipoprotein, TAGs: triglycerides, VLDL: very low-density lipoprotein

Risk ratio = Total cholesterol/ HDL-cholesterol

Values are expressed as Mean ± SEM.

Significantly different from control [a], thrombotic [b] and embolic [c] at $p<0.05$

Table 3: Pearson's correlation analysis of HIF1-α, HIF1A-AS2 and LINK-A with risk factors and biochemical markers in stroke patients.

	HIF1-α	HIF1A-AS2	LINK-A
Age	N.S	N.S	N.S
Gender	N.S	N.S	N.S
Diabetes mellitus	0.393**	0.411**	-0.266**
Hypertension	0.371**	0.445**	-0.423**
Smoking	N. S	N. S	N. S
Ischemic heart disease	N.S	N.S	-0.217*
Total cholesterol	0.527**	0.529**	-0.449**
LDL	0.379**	0.428**	-0.374**
HDL	N.S	N.S	N.S
TAGs	0.274**	N.S	N.S
VLDL	0.328**	0.297**	-0.402**
Risk Ratio	0.541**	0.531**	-0.441**
Prothrombin Time	0.281**	0.264**	-0.310**
HIF1-α	----	0.943**	-0.550**
HIF1A-AS2	0.943**	----	-0.536**
LINK-A	-0.550**	-0.536**	----
MDA	0.714**	0.687**	-0.753**
TAC	0.727**	0.707**	-0.776**
VEGF	0.596**	0.604**	-0.423**
ANG1	0.714**	0.726**	-0.700**
BDNF	-0.676**	-0.676**	0.718**

Significant correlation *at $p<0.05$, ** at $p<0.01$ and N.S non-significant.

Table 4: Positivity rates of HIF1-α, HIF1A-AS2 and LINK-A among ischemic, hemorrhagic and control groups.

Serum markers	Ischemic group (n=50) (%)	Hemorrhagic group (n=25) (%)	Control group (n=25) (%)	Chi-square X^2	*P*-value
HIF1-α					
- No. of +ve cases (≥7.19)	8 (16%)	20 (80%)	0 (0%)	29.179	0.000
- No. of -ve cases (<7.19)	42 (84%)	5 (20%)	25 (100%)		
HI1A-AS2					
- No. of +ve cases (≥7.38)	9 (18%)	20 (80%)	0 (0%)	27.015	0.000
- No. of -ve cases (<7.38)	41 (82%)	5 (20%)	25 (100%)		
LINK-A					
- No. of +ve cases (≥0.28)	3 (6%)	23 (92%)	0 (0%)	54.425	0.000
- No. of -ve cases (<0.28)	47 (94%)	2 (8%)	25 (100%)		

Table 5: Molecular markers that predict the hemorrhagic stroke using binary logistic regression.

Parameters	B	S.E.	P- value	Odds ratio	95% CI
HIF-1α	0.685	0.171	0.000	1.984	1.420-2.773
HIF1AS2	0.789	0.195	0.000	2.201	1.501-3.227
LINK-A	0.942	0.185	0.000	2.537	1.409-3.537
VEGF	0.015	0.004	0.000	1.016	1.008-1.023
ANG1	0.217	0.52	0.000	1.243	1.123-1.376

Figures

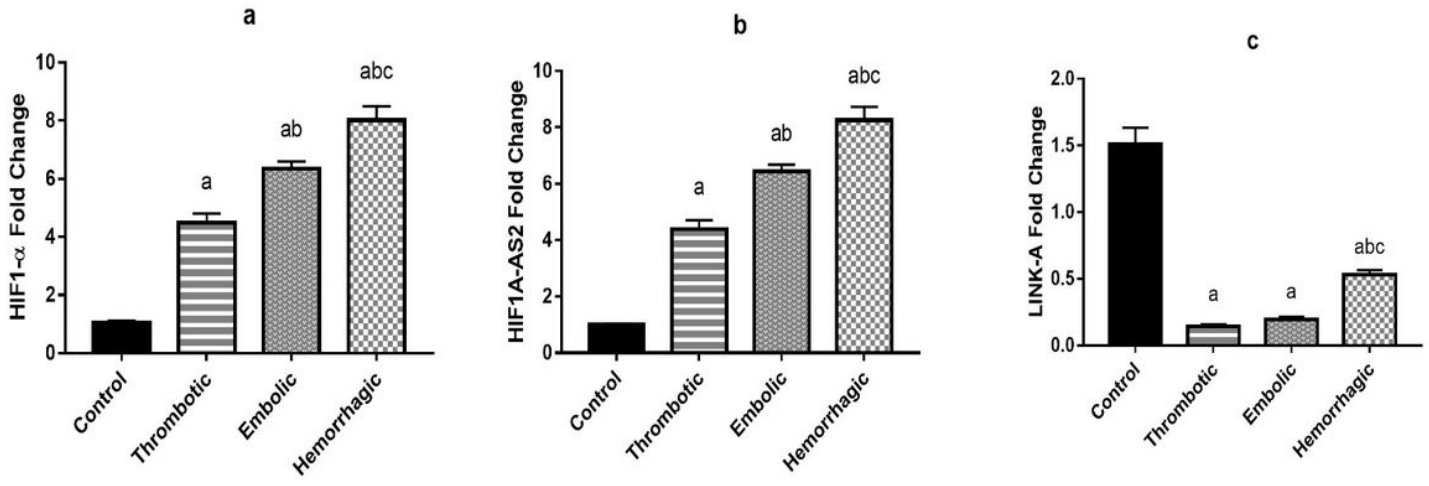

Figure 1

Expression levels of HIF1-α (a), HIF1A-AS2 (b) and LINK-A (c) in studied groups. Significantly different from control a, thrombotic b and embolic c at $p < 0.05$.

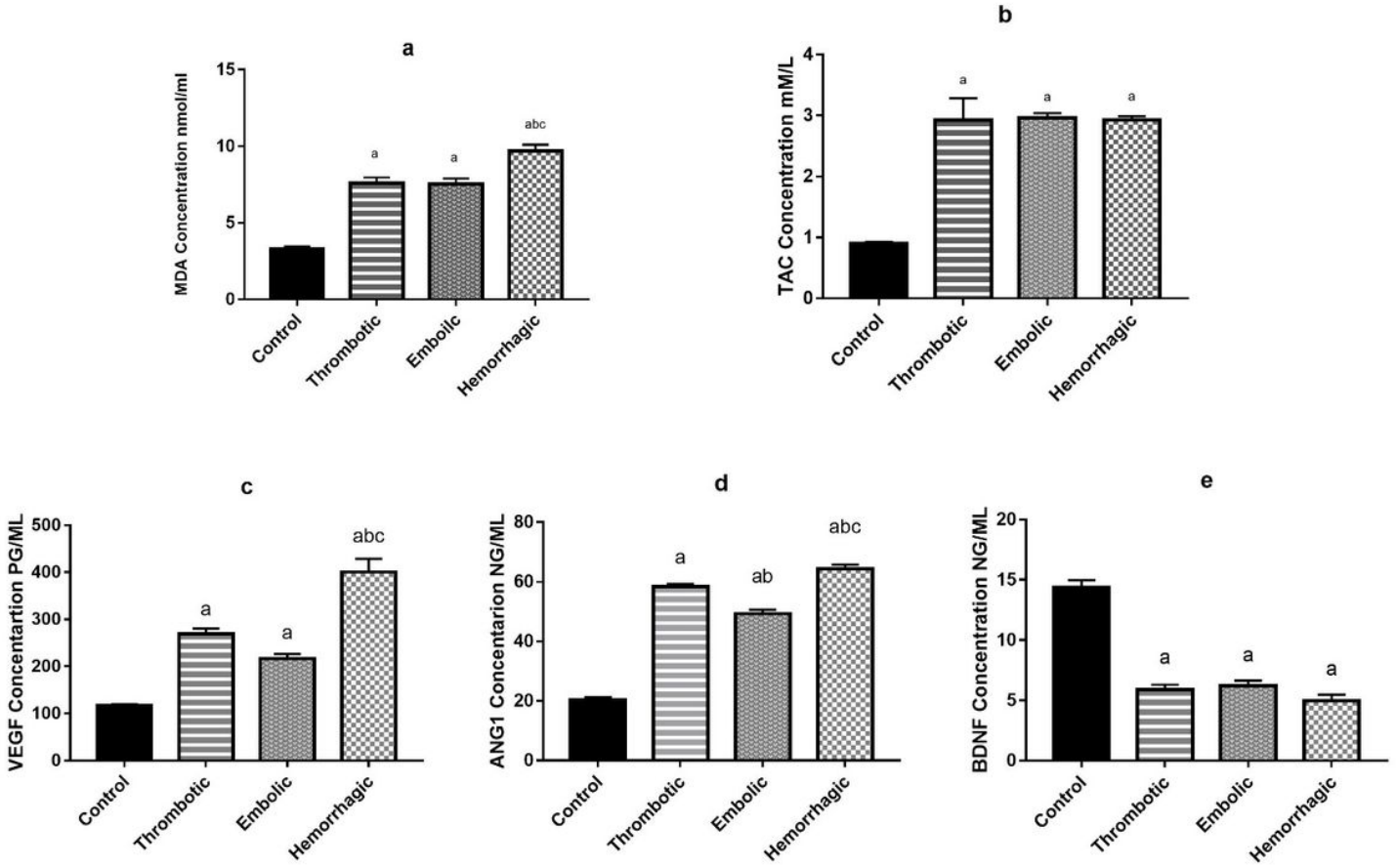

Figure 2

Serum levels of MDA (a), TAC (b), VEGF (c), ANG1 (d) and BDNF (e) in studied groups. Significantly different from; control a, thrombotic b and embolic c at p< 0.05

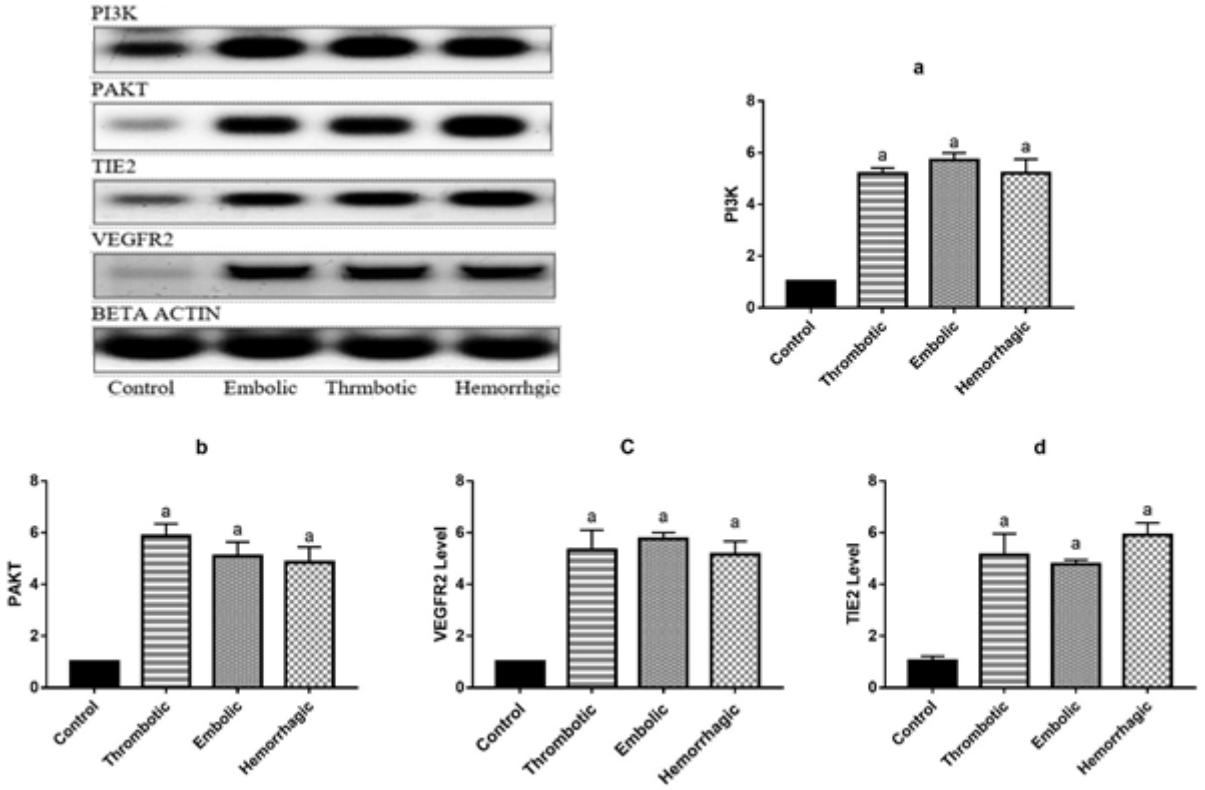

Figure 3

Protein expression levels of PI3K (a), P-AKT (b), VEGFR2 (c) and TIE2 (d) in studied groups. □ Significantly different from control group at p < 0.05.

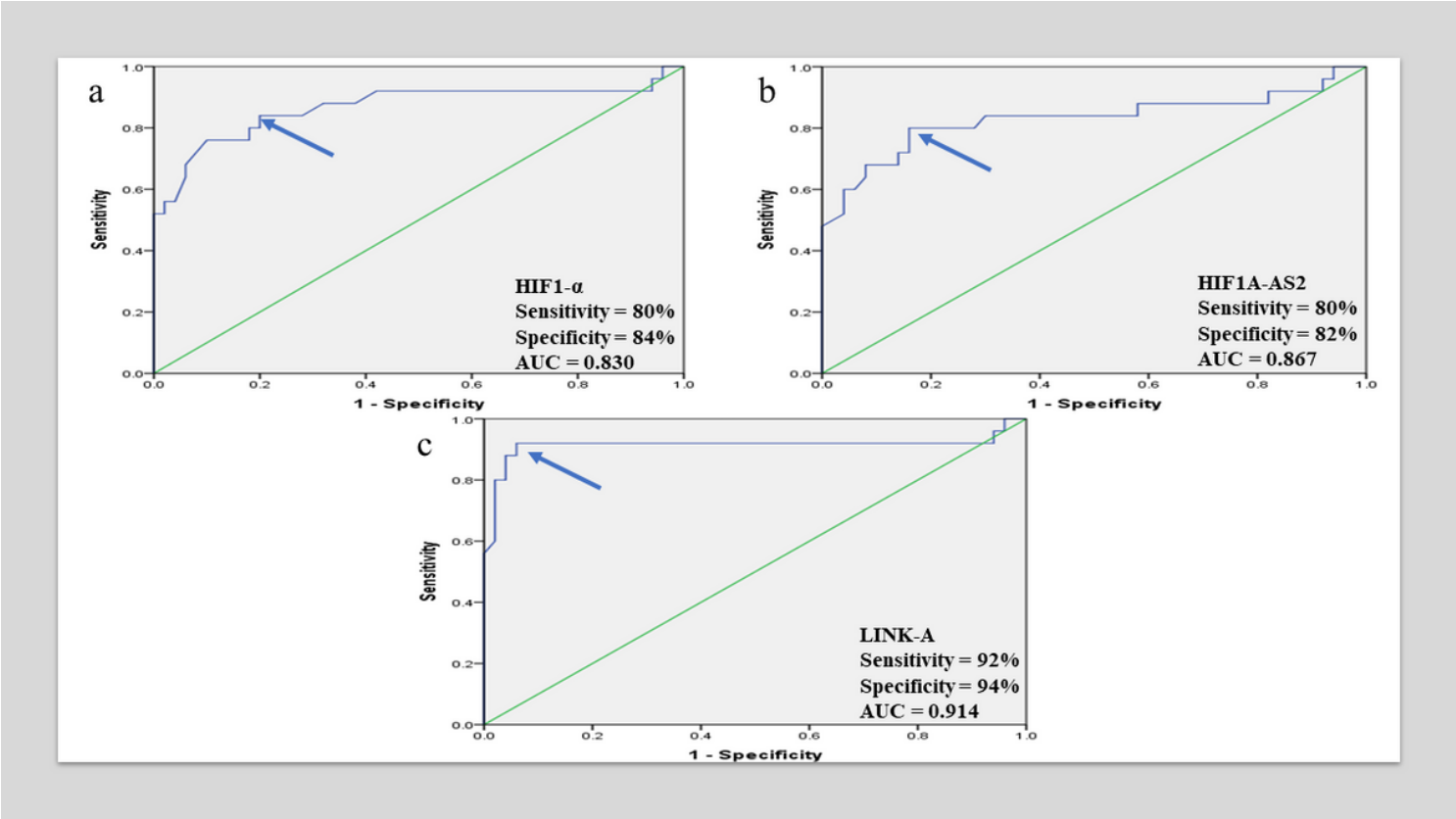

Figure 4

Receiver operator characteristic curve (ROC) of circulating HIF1-α, HIF1A-AS2 and LINK-A for predicting hemorrhagic patients from ischemic stroke patients. The arrow denotes a best cutoff point.