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RESEARCH PAPER

Correlation between Circulating Cell Free DNA and Severity of Acute Ischemic Stroke

***Nazia Tannim[1], Nowrose Jahan[2], Jahid Hasan Shovon[3], Tanjida Ruba Lita[4], Ifat Ara Begum[5], Manindronath Roy[1]**

[1]Department of Biochemistry, United Medical College and Hospital, Dhaka, [2]Department of Biochemistry, Medical College for women and Hospital, Dhaka, [3]Armed Forces Institute of Pathology, Dhaka Cantonment, Dhaka, [4]Dhaka Medical College Hospital, Dhaka, [5]Department of Biochemistry, Sir Salimullah Medical College and Hospital, Dhaka

Abstract

Background: Acute ischemic stroke management has advanced considerably, but still identifying a reliable biomarker to predict stroke severity and to optimize treatment remains challenging.

Objective: The objective of this study was to assess the correlation between plasma circulating cell free DNA (ccfDNA) and the severity of acute ischemic stroke based on National Institutes of Health Stroke Scale (NIHSS) score.

Methods: This cross-sectional study was carried out from March 2023 to January 2024, at the department of Biochemistry, Sir Salimullah Medical College, Dhaka, Bangladesh. A total 36 patients with ischemic stroke were selected based on clinical and neuroradiological findings within 72 hours of the event. At the time of admission, a clinical assessment was conducted using the NIHSS score. After extracting the ccfDNA from plasma it's purity and the quantity was initially observed using a Nanodrop spectrophotometer. Finally, to validate the extraction and quantification process, ccfDNA was again measured using TaqMan-based real-time PCR targeting the beta-globin gene, where the cycle threshold values were converted to kilogenome equivalents per liter.

Results: Patients with higher NIHSS score had larger amounts of ccfDNA (p-value <0.05). Additionally, a strong positive correlation (r = 0.643; p<0.05) was observed between the NIHSS score and the amount of ccfDNA following ischemic stroke.

Conclusion: Plasma ccfDNA level progressively rises with the severity of damage in acute ischemic stroke.

Keywords: Circulating cell free DNA (ccfDNA), acute ischemic stroke, National Institutes of Health Stroke Scale (NIHSS) score, stroke severity

Introduction

Stroke is a universal threat. It is the third most prevalent cause of death and debilitation globally.[1] According to current statistics, 16.9 million people are facing stroke annually. Although stroke especially ischemic stroke can occur at any age, it is more common in older age. Poor diet, depression and unhealthy lifestyle are the major contributing factors behind the higher prevalence of stroke in recent years.[2] The incidence of stroke has almost doubled in lower and lower middle-income nations in the past 40 years and they account for 86% of fatalities an 89% of disability adjusted life years (DALYs).[3]

***Correspondence:** Nazia Tannim, Department of Biochemistry, United Medical College and Hospital, Dhaka.*
Email: chaityafmc@gmail.com
ORCID ID: 0009-0007-4486-0853

Stroke is a complex condition characterized by focal disturbance of neuronal function lasting longer than 24 hours.[4] It can be broadly classified into ischemic stroke and hemorrhagic stroke. Among these, ischemic stroke is more frequent (85%) cause of blood clotting which ultimately limits blood from reaching into certain parts of the brain.

Till now, acute ischemic stroke is diagnosed based on clinical examination and neuroradiological imaging like computed tomography (CT scan) and magnetic resonance imaging (MRI). These diagnostic facilities are not available in most of the field level hospitals and emergency departments. Additionally, ischemic stroke is isodense in CT scan. So, initial critical decision regarding diagnosis of ischemic stroke is made on the basis of clinical examination. Many patients are frequently referred based on the clinical

examination findings which are quite expensive and further burdened by a false positive diagnosis. A meta-analysis showed that no existing scale is highly accurate in identifying ischemic stroke, with false positive rates ranging from 50% to 65%.[5] So, it is evident that only clinical examination is not sufficient and neuroradiological facilities are not always available. Blood serves as one of the most frequently used biofluids for clinical and research purpose since it is easily accessible and abundant. The main purpose of this study was to observe if there is any minimally invasive and reliable biomarker that not only can diagnose acute ischemic stroke rapidly but also can categorize the patients based on their severity. However, none of the blood marker that has been found so far has proven helpful in clinical practice due to lack of sufficient accuracy, precision, sensitivity and specificity.[1]

Circulating cell free DNA (ccfDNA) are small double stranded DNA fragments first mentioned by Mandel and Metais in 1948. These DNA fragments can be found in blood and other body fluids in both healthy and diseased individuals. In healthy individuals its level is <10 ng/ml or around 1000-3000 kilogenome equivalent/L.[6,7] Depending on the severity, its level can rise up to hundred or thousand times greater in patients with acute ischemic stroke.

Large vessel occlusion, which includes the internal carotid artery, vertebrobasilar artery or middle cerebral artery, are responsible for 11-29% of acute ischemic stroke.[8,9] Whenever ischemic stroke occurs, the area with permanent brain tissue damage is called the 'core infraction' while the surrounding area is called 'penumbra' with decreased blood supply that can be recovered with timely restoration of blood flow.[5] If the severe form of stroke can be identified, a preemptive management plan can be designed to reduce the mortality of stroke. This study hypothesized that as soon as an ischemic stroke occurs, ccfDNA swiftly enters into the circulation which can be utilized to evaluate the severity of the disease. Therefore, to observe the relationship of ccfDNA with severity of acute ischemic stroke, ccfDNA was extracted and the level was measured in all study subjects.

Materials and Methods

This cross-sectional analytical study was performed from March 2023 to January 2024after the approval of Institutional Review Board (IRB) in the department of Biochemistry, Sir Salimullah Medical College & Mitford Hospital (SSMC&MH), Dhaka, Bangladesh. Study subjects were selected based on their symptoms and signs of suspected acute ischemic stroke from emergency and in patient department of neurology, SSMC&MH. 36 patients, including both men and women aged 18 to 90 years, were enrolled in this study following clinical examination and neuroimaging with CT scan. Informed written consent was taken from all the subjects or their attendants.

Subjects who had pervious history of stroke or transient ischemic attack, end stage renal disease, chronic liver disease and malignancy were excluded from the study. Also, pregnant, lactating women and subjects who were unwilling to provide blood samples were excluded from the study.

The National Institutes of Health Stroke Scale (NIHSS) was employed to evaluate the severity of acute ischemic stroke. They were measured on a 42-point scale with 11 categories where a mild stroke was classified as having a NIHSS score <6, moderate stroke as 6–16 and severe stroke as >16.[1]

Neuroimaging using Toshiba Aquilion Prime CT scanner-160 slice was done within 72 hours of the event. Presence of ischemic stroke from proper history, clinical examination and CT scan report was verified by both expert radiologist and neurologist.

Measurement of Circulating Cell-Free DNA in Plasma

5 ml venous blood was collected in EDTA tubes and centrifuged at 2000 rpm for 10 minutes. Then plasma was removed and again centrifuged for 10 minutes at 2000 rpm. The supernatant was transferred into a clean eppendorf and stored at -30°C.

Circulating cell-free DNA (ccfDNA) was extracted from 1 mL of plasma using the Maxwell® RSC ccfDNA Plasma Kit (Promega, USA), following the manufacturer's instructions. The initial quantity and purity of the extracted DNA was checked by using a nanodrop UV spectrophotometer (Thermo Fisher Scientific, USA). The absorbance ratio at 260 nm and 280 nm (A260/A280) was used to ensure minimal contamination from proteins or other impurities.

To confirm that the extracted nucleic acid represented amplifiable human-derived DNA rather than non-specific or fragmented nucleic acids, quantitative real-time PCR (qEPCR) targeting the beta-globin gene was performed. The beta-globin gene, a single-copy housekeeping gene present in all nucleated human

cells, serves to validate the integrity and human origin of the extracted DNA. This step ensures that the measured DNA corresponds specifically to human ccfDNA and verifies the efficiency and success of the extraction process, as spectrophotometric quantification alone cannot differentiate between human and non-human nucleic acids.

Each qPCR reaction for beta globin gene included a forward primer 5'-GTGCACCTGACTCCTGAGGAGA-3' and a reverse primer of 5'-CCTTGATACCAACC TGCCCAG-3' and a fluorescent probe 5'-(VIC) AAGGTGAACGTGGATGAAGTTGGTGG (TAMRA)-3'. The fluorescence was measured during each cycle's extension step. Finally, the results were represented as kilogenome equivalent/L.

Qualitative data were expressed with percentage while the nominal data were expressed as mean ± SD. The 3 groups of NIHSS score and their plasma ccfDNA were compared using one-way ANOVA test. Post-hoc analysis using the Bonferroni test was done to look for multiple group comparisons. Pearson's correlation was employed to show the correlation between two continuous variables. p-value <0.05 was accepted as statistically significant.

Results

A total 36 patients with acute ischemic stroke were included in this study. The mean age of the study subjects was 65.86 ± 9.71 years. Among them around 66.7% were male and 33.3% were female. Their mean NIHSS score was 13.39 ± 8.74 during the time of admission (table-I).

Table I: Characteristics of the study subjects (N = 36)

Patient characteristics	Result (n=36)
Age (years)	65.86 ± 9.71
Range	49-83
Gender	
Male (%)	66.7%
Female (%)	33.3%
NIHSS admission score	13.39 ± 8.74

In patients with severe stroke (NIHSS score >16), mean ccfDNA level was 8380.94 ± 3664.66 kilogenome equivalent/L as compared to mild (5389.77 ± 2089.45 kilogenome equivalent/L) or moderate stroke (5544.17 ± 2126.10 kilogenome equivalent/L). Here, ccfDNA level increased significantly (p <0.05) with the severity of acute ischemic stroke according to NIHSS scoring system (table-II).

Table II: Comparison of ccfDNA among different severity grades of acute ischemic stroke (N = 36)

NIHSS Score	Circulating cell free DNA (ccfDNA) (kilogenome equivalent/L)		p-value
	n	Mean ± SD	
Mild stroke	6	5389.77 ± 2089.45	
Moderate stroke	13	5544.17 ± 2126.10	<0.05
Severe stroke	17	8380.94 ± 3664.66	

p-value reached form one way ANOVA test

It revealed no significant difference in ccfDNA levels between mild and moderate stroke groups (p-value >0.05). However, significant differences were observed in between mild and severe stroke (p-value <0.001) as well as between severe and moderate stroke groups (p-value <0.05) (table-III).

Table III: Comparison of plasma ccfDNA in between different severity grades of acute ischemic stroke (N = 36)

NIHSS Score	p-value
Mild stroke vs moderate stroke	1.000^{ns}
Moderate stroke vs severe stroke	<0.05
Severe stroke vs mild stroke	<0.001

ns=not significant, p-value reached by applying post-hoc analysis using the Bonferroni test to observe the difference in ccfDNA levels in between mild, moderate and severe stroke

There was a strong positive correlation (r = 0.643; p < 0.05) between circulating cell free DNA and severity of acute ischemic stroke determine by NIHSS score in all study subjects (figure 1).

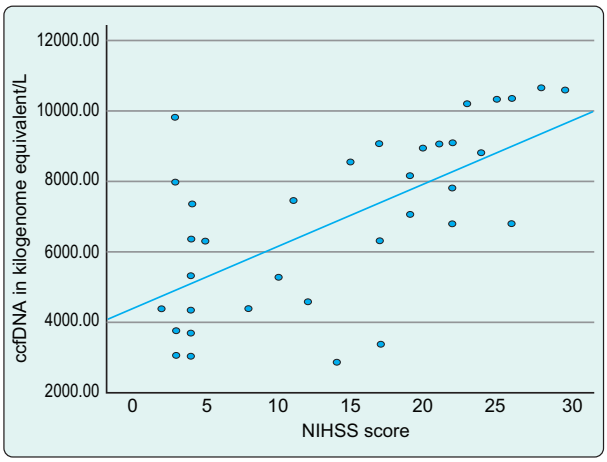

Figure 1: Scatter diagram showing correlation (r = 0.643; p < 0.05) between ccfDNA level with the severity of acute ischemic stroke (Pearson's correlation test was done)

Discussion

This study was conducted to assess the relationship of plasma ccfDNA levels with the severity of acute ischemic stroke. A total 36 subjects were clinically examined with NIHSS score and were divided into mild, moderate and severe stroke. Their mean NIHSS score was 13.39 ± 8.74 which aligns with previous findings.[10]

This analysis revealed that patients classified with severe stroke exhibited significantly higher plasma ccfDNA concentrations compared to those with mild or moderate strokes. Notably, while the difference between mild and moderate groups was not statistically significant, but the differences were significant between moderate and severe as well as mild and severe stroke. Also, a statistically significant positive correlation ($r = 0.643$; $p < 0.05$) was identified between circulating cell-free DNA (ccfDNA) levels and the severity of acute ischemic stroke. This pattern suggests a direct relationship between ccfDNA levels and the extent of neurological impairment.

During an ischemic stroke, reduced cerebral blood flow leads to a significant drop in oxygen and glucose delivery to brain tissues. This energy deficit forces cells to rely on anaerobic glycolysis, which results in lactic acid accumulation.[11] The ensuing acidic environment contributes to astrocyte dysfunction and triggers the release of pro-inflammatory cytokines. These events collectively promote necrosis and apoptosis. As the blood-brain barrier becomes compromised, it allows small DNA fragments, including circulating cell-free DNA (ccfDNA), to enter the bloodstream. Consequently, plasma ccfDNA levels are elevated in acute ischemic stroke.[12-13]

Bustamante and colleagues also identified that ccfDNA levels rise shortly after a stroke event, suggesting its potential as a biomarker for assessing the severity and predicting the prognosis of acute ischemic stroke.[14]

Building upon earlier research, Lam and colleagues (2006) proposed that plasma circulating cell-free DNA (ccfDNA) could serve as a prognostic biomarker for predicting mortality and morbidity in stroke patients, particularly when neuroimaging results are inconclusive.[15]

Plasma ccfDNA can enhance the clinical evaluation of AIS patients, aiding in the optimization of treatment plans.[16] This approach is particularly beneficial in settings with limited resources, where advanced imaging modalities may not be readily available.

Conclusion

In conclusion, this study suggests strong positive correlation between plasma ccfDNA levels and the severity of acute ischemic stroke. However, larger-scale studies are needed to confirm these findings, and future research should consider using next-generation sequencing for more detailed analysis.

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