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RESEARCH

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Quantitative analysis of cell-free plasma DNA as a prognostic biomarker in acute ischemic stroke patients

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Abstract

Background Stroke has become a leading cause of death and disability worldwide, and despite the introduction of new screening programs, therapies, and monitoring technologies, there is still a need to develop more useful biochemical tests to monitor treatment response and inform clinical decision-making. Cell-free DNA released from damaged neurons in stroke patients may be useful in assessing stroke prognosis. The purpose of this study was to evaluate the role of cell-free DNA and a highly sensitive blood biomarker in acute ischemic stroke. 188 patients with acute ischemic stroke were recruited for the study. The level of cell-free DNA in plasma was estimated using a real-time PCR assay for the β-globin gene (Qiagen-Rotor-Gene Q MDX, Germany). Clinical assessment was performed with the National Institutes of Health Stroke Scale (NIHSS) at the time of admission. After a period of three months from the onset of stroke, (mRS) scores were estimated using the modified Rankin scale.

Results Elevated levels of cell-free DNA were found in patients with higher NIHSS admission scores and mRS 3-month scores ($p < 0.05$). The regression analysis revealed that the markers are associated with an unfavorable outcome in comparison to other stroke risk factors ($R^2 = 0.224$). Stroke outcome was relatively better in patients with a cell-free DNA level < 10,000 kilogenome equivalents/L ($p < 0.05$).

Conclusions Measurement of total cell-free DNA levels is a simpler and less-expensive biomarker suggesting potential clinical application of blood-based test. Cell-free DNA can contribute to the clinical evaluation and optimal management of ischemic stroke patients. This biomarker seems to have the potential to predict the long-term prognosis of acute ischemic stroke.

Keywords Cell-free DNA, Acute stroke, Mechanical thrombectomy, Blood biomarker

Background

Stroke is one of the leading causes of morbidity and mortality worldwide [1]. According to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017, stroke is the third leading cause of death and disability. This may be attributed to the lifestyle changes associated with the increasing prevalence of modifiable risk factors for stroke. Although the diagnosis of stroke is primarily clinical, there is great interest in finding new biomarkers that can aid in the prognosis and treatment of stroke.

Many studies are still ongoing and one of their main goals is to find trustworthy, non-invasive stroke biomarkers [1–3]. Despite this, none of the biomarkers discovered to date have been useful in clinical practice, due in part to the strict criteria that biomarkers must meet before they can be used. The biomarker's accuracy, precision,

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sensitivity, and specificity for the decided outcome, as well as a standardized method for gathering the data and simplicity of interpretation, are some of these criteria [4]. In a number of conditions, including pregnancy, cancer, transplant rejection and trauma, the DNA circulating in the plasma is altered both qualitatively and quantitatively. Although the exact mechanisms by which nucleic acids are released into the circulation are still unknown, cell death is likely a critical factor. The blood–brain barrier is disrupted and cell death is associated with both ischemic and hemorrhagic stroke [5]. This study hypothesized that DNA could quickly enter the bloodstream once a stroke has started and be used to estimate the severity of the disease.

The American Stroke Association Stroke Prevention Guidelines includes a thorough review of markers of predicted stroke risk for primary and secondary prevention. The use of biomarkers in stroke diagnosis and prognosis assessment is an emerging and rapidly developing area [5, 6]. The initial evaluation of stroke patients included a detailed history and physical examination, followed by brain imaging and laboratory tests. Neuroimaging is commonly used to aid in stroke diagnosis, determine event severity, and predict functional outcome. However, imaging has some limitations. Computed tomography (CT) is often unremarkable in patients with mild ischemic stroke. Magnetic resonance imaging (MRI) is more sensitive in detecting ischemia but may not be available at all centers. MRI cannot be performed on acutely ill patients who are restless or have implants. Therefore, there is a need to evaluate blood markers for acute cerebral ischemia, which can be helpful in the prognosis of stroke patients and thus in the overall management of acute stroke. In the current study, researchers evaluated the clinical use of cell-free DNA to assess the severity and long-term prognosis of acute ischemic stroke.

Methods

One hundred and eighty eight patients presenting to the emergency department of our comprehensive stroke center from December 21, 2019 to September 12, 2021 with an acute ischemic stroke were consecutively enrolled in this study. All included patients had a proximal M1-MCA occlusion with a similar risk factor distribution.

Exclusion criteria: Cases with trauma, meningitis, encephalitis or other systemic infections, hypertensive encephalopathy, intracranial tumors, migraine, post-cardiac arrest, drug overdose, organ failure, psychiatric syndromes, shock, and patients presenting late, d. H. > 24 h after symptom onset.

Radiological and clinical examination: Clinical evaluation was followed by basic laboratory and imaging studies, including CT or MRI, to determine the exact nature of the stroke-like syndrome. Depending on the etiopathogenesis, the cases were roughly classified as infarction or hemorrhage and only patients with ischemic stroke were considered for this study.

Neurologic Outcome Scale Assessment: The National Institutes of Health Stroke Scale (NIHSS) was used to clinically assess stroke severity. It is a 42-point scale that quantifies neurological deficits into 11 categories, such that normal function with no deficit is scored as zero. An NIHSS score of 6 was considered mild stroke, while a score > 16 was considered major stroke [2]. Neurological outcome was measured 3 months after onset of symptoms using modified Rankin scale (mRS). The mRS is a functional rating scale used to assess neurological deficits, with a score of zero indicating the absence of symptoms, while a score of 5 indicates severe disability. mRS score of 0–2 was considered a good result, while 3–6 was considered a poor result [3].

DNA extraction, quantification, and PCR amplification

Five ml of a venous blood sample was collected in EDTA tubes in the hospital emergency department and centrifuged at 14,000*g* for 20 min. Supernatant plasma was removed without disturbing the buffy coat and stored at − 80 °C until further processing. Cell-free DNA was extracted from 1 ml of plasma using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany). The amount of DNA extracted was assessed using a Nanodrop spectrophotometer (Thermo Scientific). Cell-free DNA estimation was performed by real-time quantitative PCR assay for globin gene (Quanti Nova Probe RT-PCR Kit – Qiagen) on Rotor-Gene Q platform (Qiagen, Germany). The β-globin gene PCR system consists of the amplification primers β-globin gene 354F (5-GTG CAC CTG ACT CCT GAG A-3) and β-globin gene -455R (5-CCT TGA TAC CAA CCT GCC CAG-3) and a dual-labeled fluorescent PCR probe β-globin-402 T [5-(VIC) AAG GTG AAC GTG GAT GAA GTT GGT GG-3] [7]. The time required for the estimation of cell-free DNA is about 3 h, which will decrease with further technological advances. Real-time PCR quantitative results were expressed as cell-free DNA kilogenome equivalents/L. The mean plasma DNA concentration in the control group was 3154 kilogenome equivalents/l [8].

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, Chicago, USA). Continuous variables are presented as mean standard deviation. Student's *t* test was used to compare test scores

Table 1 Patients characteristics

Patients characteristics	Results ($n=188$)
Age (years) Mean (range)	61.28 (26–90)
Gender—male (%)	128 (68.51%)
Risk factors-number of patients (%)	
Hypertension	94 (50%)
Diabetes mellitus	35 (18.51%)
Ischemic heart disease	28 (15%)
Hyperlipidemia	75 (40%)
Tobacco chewing	28 (15%)
Smoking	44 (23%)
Alcoholic	56 (30%)
Duration in hours[#] (range)	10.74 (1–12)
Large-artery atherosclerosis	38 (20%)
Cardio-embolism	57 (30%)
Small-artery occlusion	65 (35%)
Stroke of other determined etiology	28 (15%)
Stroke of undetermined etiology	37 (20%)
Therapeutic intervention	92 (49%)
Mechanical thrombectomy	54 (58%)
IV thrombolysis	57 (62%)
Cell-free DNA median* (range)	8786 (902–33,135)

IV thrombolysis intravenous thrombolysis

[*] Kilogenome equivalents/L

[#] Time between onset of stroke symptoms and blood collection (median time)

Table 2 R-Value of clinical assessment scale scores

	Mean ± (SD) (range)	*r*-values
NIHSS admission	15.11 ± 8.28 (0–42)	0.235
mRS 3 month	2.54 ± 1.62 (0–6)	0.395

between groups. Correlations were determined using Spearman's or Pearson's tests. Descriptive statistics and data comparison tests (Mann–Whitney) were performed. All tests were two-tailed and statistical significance was determined by p values <0.05.

Results

The baseline characteristics of stroke patients and the cell-free DNA levels did not correlate significantly. The demographic data are summarized in (Table 1). All included patients had a proximal M1-MCA occlusion with a similar risk factor distribution and an intervention according to standard guidelines for IVT with t-PA and mechanical thrombectomy.

There was a correlation between the Cell-free DNA levels and NIHSS admission as well as the mRS 3-month scores (Table 2).

Table 3 Significance of cell-free DNA > 10,000 kilogenome equivalents per liter with NIHSS admission score and mRS 3-month score

	p-values
NIHSS < 6	0.03
NIHSS 6–16	0.81
NIHSS > 16	0.01
mRS 3 Month (< 3)	0.002

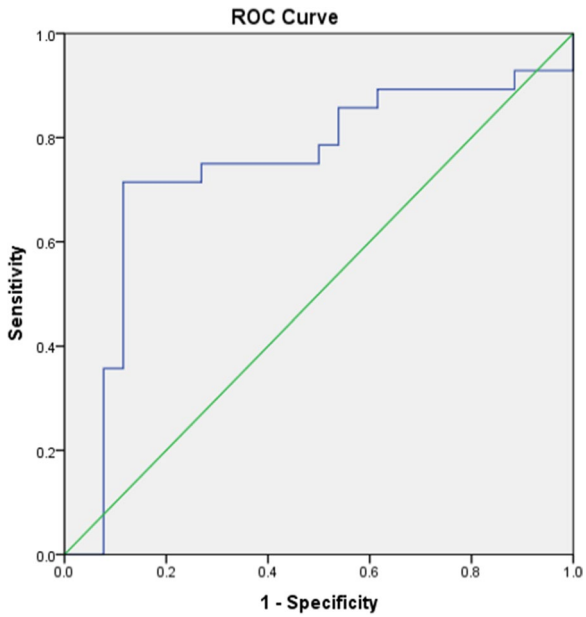

Fig. 1 ROC curve analysis of cell-free DNA concentrations and MRS 3-month score for the prediction of stroke prognosis. Values indicated on the *X* (MRS 3 month score) and *Y* axes (Cell-free DNA level) are expressed in percentages. The area under the curve was 0.74 (95% CI 0.44–0.80 $p=0.002$) at 72% sensitivity and 88% specificity

The p value was significant, which means that the severity of the NIHSS scores at admission and outcome measured by the mRS 3-month score were associated with higher levels Cell-free DNA (Table 3).

With optimal ROC curve analysis of cell-free DNA concentrations and MRS 3-month score to predict stroke prognosis, the values on the x (MRS 3-month score) and y-axis (cell-free DNA level) are expressed in percent. The area under the curve is 0.74 (95% CI 0.44–0.80 $p=0.002$) with 72% sensitivity and 88% specificity (Fig. 1).

The p value was also significant for cell-free DNA > 10,000 kilogenome equivalents per liter when the clinical outcome was measured by mRS at 3 months (Fig. 2).

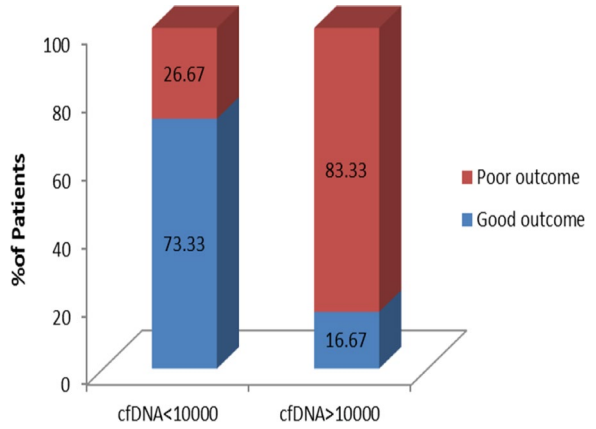

Fig. 2 Significance of cell-free DNA > 10,000 kilogenome equivalents per liter when the clinical outcome was measured by mRS at 3 months (*p* value = 0.014)

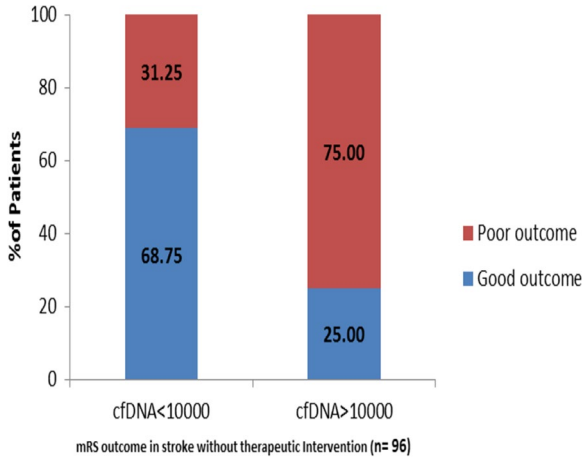

Fig. 4 Comparison of mRS 3 month outcome without therapeutic intervention ($P < 0.05$)

The 92 patients underwent mechanical thrombectomy or IVT according to standard American Stroke Association guidelines [9]. These were associated with an improved outcome as measured by the mRS 3-month score ($P < 0.05$) (Figs. 3, 4). Patients who underwent IVT and mechanical thrombectomy or only mechanical thrombectomy achieved mTICI grade 2b or 313 in all cases. Further analysis at the cell-free DNA level below 10,000 kilogenome equivalents per liter showed a better outcome at three months ($P < 0.05$) (Figs. 3, 4).

Taken together, these observations suggest that circulating cell-free DNA levels may be useful for prognostication of AIS early in the acute phase of treatment.

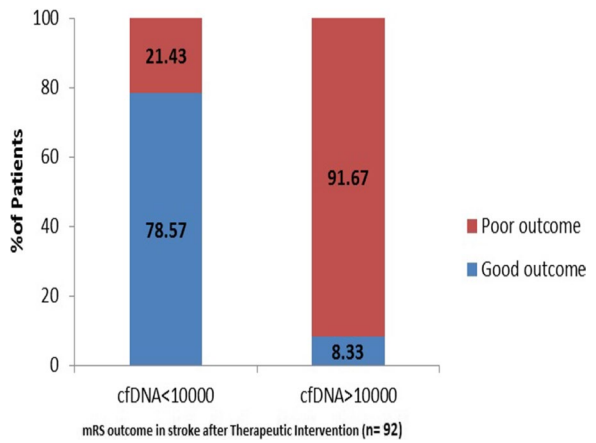

Fig. 3 Comparison of mRS 3 month outcome after therapeutic intervention ($P < 0.05$)

Discussion

This is the first study to show that patients' circulating plasma DNA levels increase within the first 24 h after an acute stroke, as determined by real-time PCR analysis of β-globin gene levels. Furthermore, we have shown that plasma DNA measurements can be helpful for early risk classification and prognosis of acute hospital care. The aim of this study was to evaluate the ability of cell-free DNA levels to detect prognosis of ischemic stroke early in the acute treatment phase and to investigate the relationship between cell-free DNA levels and stroke-induced neurological outcomes. Our results suggest that cf-DNA may be helpful in identifying AIS in acute care settings. There is not yet a proposed stroke biomarker that is regularly used in the clinical setting. A number of studies have examined cell-free DNA moving through body fluids during stroke as a potential diagnosis of this condition [10, 11]. Imaging helps identify the etiology of acute ischemic stroke and predict prognosis in patients after an initial clinical assessment. Despite the robustness of the technology, some individuals may be overlooked because clinical investigations are illogical and results are occasionally difficult to predict. In addition, in circumstances where it is urgent, the NCCT scan may not depict the full extent of the brain damage [12]. Modern imaging procedures diagnose a stroke safely and reliably and provide information to predict the outcome. However, some patients with clinical stroke have no detectable abnormality on neuroimaging, and other patients are unsuitable for such investigations [13]. Therefore, this study demonstrated the potential of cell-free plasma DNA levels to predict mortality and morbidity after stroke in patients with negative neuroimaging results. A peripheral blood biomarker should not only be specific

and sensitive, but also be readily available and inexpensive. Several biomarkers have been identified to diagnose and characterize stroke. However, interpretation of most of those markers is confounded by multiple factors, such as latent rise, effect of blood–brain barrier, and diverse etiopathogenesis [4, 10]. A major challenge that remains to date is finding a solution to these issues so that blood markers can add value to the information received through clinical evaluation and neuroimaging. Therefore, this study was designed to evaluate the status and usefulness of cell-free DNA in patients with early-phase acute stroke within 24 h of the event, with a focus on its use as an aid in assessing stroke severity and prognosis. In an ischemic stroke, an obstruction in arterial flow causes a cerebral infarction. Stroke-associated brain damage is ultimately due to a lack of metabolic substrates. Neurons have a high energy requirement, which is covered by aerobic metabolism. Therefore, rapid depletion of oxygen and glucose supplies in stroke leads to cessation of oxidative phosphorylation in neurons. The ongoing inflammatory and oxidative process accelerates neuronal damage and eventually leads to apoptosis [10]. Although the actual mechanisms by which nucleic acids are released into the circulation are unknown, cell death is probably a significant factor [7, 8].

Cellular ischemia and tissue infarction can lead to release of DNA from neuronal cells [6, 8]. There are two forms of DNA in circulation: free DNA and DNA–protein complexes. The former, which is present in the free form in plasma, has a short half-life of about 10–15 min due to rapid hepatic and renal clearance [14]. However, we found that those with a severe clinical presentation and poor outcome had higher levels of cell-free DNA. The clinical presentation of stroke and its outcome are influenced by a number of factors.

Similar to these results, we also found higher levels of cell-free DNA in poor-outcome cases, as measured by the mRS score at 3 months. Chronic inflammatory processes associated with traditional risk factors for atherosclerosis, such as aging, hypertension, diabetes, dyslipidemia, and smoking, play an important role in the development of stroke. Therefore, the association between inflammatory markers, such as cell-free DNA and stroke development, may contribute to a better understanding of stroke pathogenesis. It will be interesting to carry out serial measurements of cell-free DNA in stroke patients in order to be able to better analyze their influence on the long-term prognosis.

Rainer and colleagues found a correlation between plasma cell-free DNA concentrations and stroke severity and suggested that this could be used to predict emergency department outcome. The same group also showed that it is a better marker of hemorrhagic stroke [6].

Lam and colleagues proposed plasma cell-free DNA as a prognostic marker to predict mortality and morbidity after stroke in stroke patients with negative neuroimaging findings within the first 24 h of symptom onset [13]. Bustamante and colleagues suggested that cell-free DNA could be a surrogate marker for monitoring the effectiveness of IV thrombolysis by predicting short-term neurological outcomes [15]. Cell-free DNA would be released into plasma soon after the onset of a stroke and could be useful in assessing disease severity and prognosis.

Vajpeyee and colleagues suggested that cell-free DNA concentration correlates well with stroke intervention outcome in acute ischemic stroke patients. Long-term care and rehabilitation place a significant financial burden on families of stroke survivors.

The number of stroke survivors is increasing as modern treatment modalities and stroke units become more widely available. To make best use of resources, blood biomarkers should be evaluated to predict neurological outcome in such patients. To the best of our knowledge, this is the first study to hypothesize that cell-free DNA can be used as a marker to monitor disease progression and assess outcome in stroke patients after an intervention [16, 17].

Therefore, the results of our study were not entirely unexpected and consistent with data published in the literature. Therapeutic interventions in the form of mechanical thrombectomy or intravenous thrombolysis were performed in 92 cases according to standard guidelines. These patients had better outcomes when cell-free DNA was < 10,000 kilogenome equivalents/L (ROC cutoff Fig. 2) as assessed by the mRS 3-month score ($p < 0.05$).

Therefore, relatives of patients with high initial cell-free DNA, i. H. > 10,000 kilogenome equivalents/L, warned of the limited chances of favorable outcomes after the procedure. This will help them make informed decisions before committing to any interventions.

Thus, cell-free DNA can complement clinical assessment of stroke patients to predict outcome stratification, which can help optimize treatment strategies. However, because measuring cell-free DNA is simple, fast, and inexpensive, it may be preferred by physicians to assess stroke severity and prognosis, particularly in resource-constrained locations.

Limitations

However, the traditional method of profiling cell-free plasma DNA for clinical use is expensive. Our method, RT-PCR quantification for the β-globin gene represents a viable option for clinical use. Recently, in 2020, the U.S. Food and Drug Administration (FDA) approved the first liquid biopsy NGS diagnostic tests for commercial use. These diagnostic tests identify mutations

in different genes in patients eligible for specific treatments, the full list of approved nucleic acid tests, including cf-DNA tests, can be viewed on the FDA website.

In addition, specific quantification of cell-free DNA with associated co-morbidities and patient outcomes in long-term follow-up can lead to a more specific response. The final infarct volume was not measured; however, this parameter could not be used for further adjustments. Some other parameters reflecting similar information, such as the NIHSS baseline, were used to fit the model.

Therefore, further studies with a larger sample size and longer follow-up in correlation with more appropriate clinical outcome measurements are needed to validate cell-free DNA quantification as an accurate and reproducible tool to measure AIS severity and outcome in real time. However, our results are still preliminary in nature and future studies are needed to replicate these results.

Conclusions

This study suggests that cell-free DNA quantification can be used as a screening tool to identify individuals with severe ischemic stroke in the acute care setting. The development of new models containing multiple markers simultaneously is advisable to achieve better sensitivity and specificity for assessing stroke prognosis. The relationships between these indicators and clinical outcomes in stroke patients require larger studies.

Abbreviations

Cf DNA	Cell-free DNA
GBD	Global Burden of Diseases
PCR	Polymerase chain reaction
ROC	Receiver operating characteristic
CT	Computed tomography
MCA	Middle cerebral artery
AUC	Area under receiver operating characteristic curve
IVT	Intravenous thrombolysis
t-PA	Tissue-plasminogen activator
MRI	Magnetic resonance imaging
mRS	Modified Rankin, Scale
GCS	Glasgow Coma Scale
NIHSS	National Institutes of Health stroke scale,
SPSS	Statistical Package for Social Sciences
SD	Standard deviation

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Author contributions

AV, ST, and MV implemented the study. ST, LBY, and PM performed data collection and sample collection. ST and LBY performed data analyses and data interpretation. AV, MV, ST, PM, and LBY revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data available upon reasonable request to the corresponding author.

Declarations

Ethics approval and consent to participate

The institutional ethics committee of Pacific Medical University & Hospital board approved the use of human subjects for this study with the reference number (PMU/IEC/186/B/2019/20). Informed, written consent was obtained from either the patient or a family member.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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