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Basic Study

Cell-free mitochondrial DNA quantification in ischemic stroke patients for non-invasive and real-time monitoring of disease severity and outcome

Cell-free mitochondrial DNA in ischemic stroke

Nusrath Fathima, Sandhya Manorenj, Sandeep Kumar Vishwakarma, Aleem Ahmed Khan

Abstract

BACKGROUND

Acute ischemic stroke (AIS) is one of the major causes of continuous increasing rate of global mortality due to the lack of timely diagnosis, prognosis and management. This study provides a primitive platform for non-invasive and cost-effective diagnosis and prognosis of patients with AIS using circulating cell-free mitochondrial DNA (cf-mtDNA) quantification and validation.

AIM

To evaluate the role of cf-mtDNA as s non-invasive, and affordable tool for real-time monitoring and prognosticating AIS patients at onset and during treatment.

METHODS

This study enrolled 88 participants including 44 patients with AIS and 44 healthy controls with almost similar mean age group at stroke onset, and at 24hrs and 72hrs of treatment. Peripheral blood samples were collected from each study participant and plasma was separated using centrifugation. The cf-mtDNA concentration was quantified using nanodrop reading and validated through RT-qPCR of ND1 relative transcript expression levels.

RESULTS

Comparative analysis of cf-mtDNA concentration in patients at disease onset showed significantly increased levels compared to control individuals for both nanodrop reading, as well as ND1 relative expression levels ($p<0.0001$). Intergroup analysis of cf-mtDNA concentration using nanodrop showed significantly reduced levels in patients at 72hrs of treatment compared to onset ($p<0.01$). However, RT-qPCR analysis showed significant reduction at 24hrs and 72hrs of treatment compared to the disease onset ($p<0.001$). The sensitivity and specificity was relatively higher for RT-qPCR than nanodrop-based cf-mtDNA quantification. Correlation analysis of both cf-mtDNA

concentration as well as ND1 relative expression with NIHSS score at baseline showed positive trend.

CONCLUSION

In summary, quantitative estimation of highly pure cf-mtDNA provides a simple, highly sensitive and specific, non-invasive, and affordable approach for real-time monitoring and prognosticating AIS patients at onset and during treatment.

Key Words: Cell-free mitochondrial DNA; ND1; ischemic stroke; circulating biomarker; NIHSS score; stroke assessment; severity and outcome

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Core Tip: Several blood biomarkers have been studied to determine the severity and outcome of ischemic stroke with limited applications. Hence, we need to establish molecular markers which can provide more comprehensive information of the stroke pathophysiology and treatment response. Dynamic quantification of plasma cell-free DNA appears to be a valid and reliable option. This research compared the real-time expression of cell-free mitochondrial DNA (ND1 gene) in ischemic stroke patients with healthy controls, and studies its prognostic value during the treatment. This study could aid in the development of clinical values for assessing real-time, non-invasive mode of ischemic stroke status in future.

INTRODUCTION

Stroke is one of the leading causes of morbidity and mortality worldwide [1]. Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) 2017 has reported stroke as the third major cause of death and disability [2]. According to the World Health

Organization (WHO), the effective prevention approaches of stroke involves decreasing the risk of hypertension, lipid levels, fasting plasma glucose levels, smoking, reduced physical activity, unhealthy diet, and high body-mass index [BMI]), which is similar to the observations from GBD 2016[3] and GBD 2017[4]. The findings of GBD 2019 Stroke Collaborators indicate that the majority of the global stroke burden (86% of deaths and 89% of disability-adjusted life-years [DALYs]) persists in lower-income and lower-middle-income countries [5]. Globally, over the past three decades, the number of stroke-related DALYs have increased substantially (by 33·5 million, from 91·5 million in 1990 to 125 million in 2019).

Emergency stroke treatment depends on the type of stroke, either ischemic or hemorrhagic. The hemorrhagic stroke treatment depends on controlling the amount of bleeding and reducing pressure in the brain caused by the excess fluid. While, intravenous administration of tissue plasminogen activator (TPA) remains the gold standard treatment for ischemic stroke [6]. Although progress in treating acute ischemic stroke has been slow over the last decade, the goals for treating this frequent condition have grown. The therapeutic window should be increased from 3 to 6hrs to improve the therapeutic efficacy because small proportion of patients arrives for treatment after three hours. Hence, the beneficial clinical outcome of stroke patients relies on examining the proportion of patients receiving timely and effective diagnosis.

Recently several studies have demonstrated that blood circulating cell-free DNA (cfDNA) analysis can efficiently distinguish ischemic stroke patients from other type of strokes or healthy population [7, 8]. CfDNA is extremely fragmented and circulates freely in blood. As a result, cfDNA can be quantified easily in blood through standard blood DNA extraction methods [9]. Blood has been one of the most widely used biofluids for both clinical and research purpose, mainly due to its abundance and easy access; however, both plasma and serum are considered reliable sources due to their stability after long-term storage at ultra-low temperatures (-20°C or -80°C). Under normal physiological conditions, small DNA fragments can cross the blood-brain barrier (BBB) and reach into plasma or serum [10]. However, pathological conditions may cause

disruption of BBB which increases its permeability and allows the open flow of several molecules, cells, and DNAs between the central nervous system (CNS) and the peripheral circulation [10]. Therefore quantification of cfDNAs in circulation can reflect pathological processes that occur in the CNS [9, 11, 12]. Although several studies have reported significance of cfDNA quantification in distinguishing stroke patients [13], none of the studies have evaluated its role before and after treatment with TPA or any other mode of acceptable treatments. Further, blood cfDNA has not traditionally been considered an ideal test for a condition like stroke [14].

The above mentioned limitations are accompanied by total DNA estimation in circulation which includes both nuclear and mitochondrial DNA. Accumulating evidences have shown that cell free-mitochondrial DNA (cf-mtDNA) are released from damaged mitochondria into circulation which act as damage-associated molecular patterns (DAMPs) with inflammation in several pathological conditions [15, 16]. Moreover, alterations in mitochondrial dynamics affect energy metabolism and post-stroke neuronal function by regulating the number, morphology, and function of mitochondria. The increased interest in mapping several crucial pathways linking stress, mitochondria, and pathophysiology is fueled by recent discoveries implicating mitochondrial signaling in cellular and physiological stress management and mental health [17].

Although cf-mtDNA haplogroups have been demonstrated for their association with stroke onset, the impact of other differential role of cf-mtDNA on stroke outcomes remains unclear. Hence, individual quantification of cf-mtDNA in circulation may provide more clear and sensitive tool to overcome current dilemma of cfDNA in stroke patients [18, 19]. However, an ideal clinical evaluation of cfDNA using a simple procedure with good predictive values is required which could be utilized in both prehospital and emergency departments. More importantly, early and accurate diagnosis of stroke patients specifically with ischemic stroke using cf-mtDNA is critical for early access to interventional therapy and to increase the likelihood of a favorable outcome.

Hence, in this study, quantitative estimation of circulating cf-mtDNA has been performed and validated through quantifying mitochondrial oxidative phosphorylation gene, NADH-ubiquinone oxidoreductase chain 1 (ND1) expression analysis in plasma samples of patients with ischemic stroke. Furthermore, the patients with onset of ischemic stroke have been differentiated from control population using cf-mtDNA quantity and relative expression levels of ND1 before and after 24hrs and 72hrs of treatment with either TPA or anti-platelet medications. The diagnostic and prognostic significance of both cf-mtDNA concentration and relative expression levels of ND1 has been evaluated and correlated among different category of stroke patients. The results of this study findings may provide a crucial early and non-invasive tool for diagnosis and prognosis of ischemic stroke patients in real-time for tracking clinical response and improved clinical management.

MATERIALS AND METHODS

2.1 Study participants

All study procedures were carried out with the approval of the Institutional Review Board of Deccan College of Medical Sciences, Hyderabad. Informed consent forms were collected from each study participants. Our study group consisted of patients with acute ischemic stroke (AIS) who were admitted to the stroke unit of our centre within the window period of 4.5hrs after onset of the stroke related symptoms. A total of 88 individuals including 44 patients with AIS (27 men and 17 women) and 44 healthy controls (29 men and 15 women) with mean age of 57.46±13.16 years for AIS and 55.46±11.13 years for healthy control were enrolled. All patients underwent a complete analysis of their neurological assessment. Stroke severity was assessed at the time of admission (referred as baseline) using the National Institute of Health Stroke Scale (NIHSS) [20]. We included only those patients who showed positive treatment response to either TPA or to anti-platelet therapy. All other patients who were non-responsive to these treatments were excluded. Further, patients with encephalitis, multiple trauma, sepsis, meningitis, hypertensive encephalopathy, migraine, intracranial tumor, post-

cardiac arrest, organ failure, endocrine disorders, psychiatric syndromes, shock with hypoperfusion, or drug overdose were also excluded. We also excluded those patients in which the time from the symptom onset to blood collection was more than 12hrs.

2.2 Sample collection and extraction of circulating cell-free mtDNA

A total of 2mL of venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-coated vacutainers from control individuals, and patients with AIS within 2hrs of window period (i. e., within 4.5hrs referred as disease at onset), and after treatment at 24hrs and 72hrs. To ensure cell-free plasma collection, EDTA vacutainers were centrifuged at 3000rpm for 15min at room temperature. The circulating cf-mtDNA was isolated from 300µL of plasma sample using an in-house protocol established in our laboratory. Briefly, 300µL of plasma was mixed with 1mL of mitochondrial isolation buffer (pH 7.8) and centrifuged at 3000rpm for 10min at 4^{o}C. After centrifugation, supernatant was collected and again centrifuged at 10,000xg for 10-15min to collect the pellet containing mitochondria. The cf-mtDNA was extracted using in-house rapid DNA extraction procedure, and precipitated using cold 100% ethanol. Finally, precipitated DNA pellet was air dried, and dissolved in 1X Tris-EDTA solution. The extracted cf-mtDNA was stored at -20^{o}C and used for quantification using nanodrop reading, and for mitochondrial DNA specific PCR of ND1 gene transcript.

2.3 Quantitative analysis of cell-free mtDNA

2.3.1 Nanodrop reading

The extracted cf-mtDNA was quantified using nanodrop reader at 260/280nm absorbance. A 260/280 ratio of approximately 1.8 and less than 2.0 was considered for pure DNA content without protein contamination and used for further analysis. The DNA concentration was recorded for each sample and reported in ng/µL.

2.3.2 RT-qPCR analysis

Cell-free mtDNA concentration was further validated using SYBR-Green based real-time quantitative polymerase chain reaction (RT-qPCR). Plasma cf-mtDNA was quantified for ND1 gene in a CFX-96 Real time system (1000^{Tm} Thermal cycler, BIORAD) using gene specific primers (forward: 5′-CTACTACAACCCTTCGCTGAC-3′

and reverse: 5′-GGATTGAGTAAACGGCTAGGC-3′). Mitochondrial 12S gene specific primers were used as endogeneous control. The RT-qPCR conditions were 94^{o}C for 3 minutes, 94^{o}C for 30 s, 54^{o}C for 30 s, and 72^{o}C for 30 s for 40 cycles along with melt-curve analysis. All the reactions were performed in triplicates in two different cohort studies with 100% PCR efficiency.

2.3.3 Correlation analysis

Correlation was established between cf-mtDNA concentration as well as ND1 relative fold expression in controls and patients at disease onset, and during the treatment at 24hrs, 48hrs, and 72hrs using by computing Pearson correlation coefficient (r) and identifying p values. Further, correlation of cf-mtDNA concentration as well as ND1 relative fold expression was performed with NIHSS score at the baseline in patients with AIS during the disease onset to predict its clinical significance.

3. Statistical analysis

The statistical analysis was performed and data was recorded using GraphPad Prism software (version 5.0, and 8.4.2). The data were presented as Mean±standard deviation (SD). One-Way Analysis of Variance (ANOVA) was used to compare the multiple groups, and student t-test was used to compare two groups with significance level $p<0.05$ at 95% confidence interval (CI). Relative operative curve (ROC) analysis was performed to predict diagnostic value of cf-mtDNA concentration and ND1 expression in relative fold change of ND1 gene which was calculated using $2^{-\Delta\Delta Ct}$ method[21]. Correlation analysis was performed by calculating Pearson correlation coefficient (r) among different groups. The p value was also calculated at 95%CI to demonstrated statistical significance of positive and negative correlation between different groups.

RESULTS

4.1 Discriminative analysis and diagnostic value of cf-mtDNA concentration

Comparative analysis of cf-mtDNA concentration in patients at disease onset showed significantly increased levels compared to control individuals (mean difference (MD), -14.16±1.691; CI, -17.55 to -10.76; $p<0.0001$; **Figure 1A**). Further, comparison of cf-

mtDNA concentration at 24hrs (MD, 8.292±2.146; CI, -12.65 to -3.933; p<0.0001; **Figure 1B**) and 72hrs (MD, 3.035±0.6559; CI, -4.378 to -1.692; p<0.0001; **Figure 1C**) with control individuals also showed significant increased values. ROC analysis of cf-mtDNA concentration in patients at disease onset showed significantly higher predictive value (AUC, 0.9808; p<0.0001; **Figure 1D**) representing 69.71% sensitivity and 76.34% specificity. Likewise, the ROC at 24hrs of treatment also showed significantly higher diagnostic value of cf-mtDNA concentration with 64.68% sensitivity and 55.91% specificity (AUC, 0.7290; P = 0.029; **Figure 1E**); however, it represented 25.18% poor diagnostic value to discriminate patients according to their response to treatment at 24hrs. In contrast, at 72hrs of treatment cf-mtDNA didn't show positive predicative value for the diagnostic significance and represented only 71.42% sensitivity and 50.18% specificity (AUC, 0.7500; P = 0.1128; **Figure 1F**).

4.2 Intergroup analysis and diagnostic significance of cf-mtDNA concentration

Intergroup analysis showed significantly reduced levels of cf-mtDNA concentration in patients at 72hrs of treatment compared to the disease onset (MD, 3 11.12; CI, 0.7827 to 21.46; p<0.01; **Figure 2A**). However, patients after 24hrs of treatment didn't show significant difference in patients at onset (MD, 5.864; CI, -1.059 to 12.79; p>0.05). Similarly, no significant difference was observed between 24hrs and 72hrs of treatment (MD, 5.257; CI, -5.982 to 16.50; p>0.05). The ROC analysis of cf-mtDNA concentration at 24hrs of treatment showed significantl higher predictive value (AUC, 0.7115; p<0.044; **Figure 2B**) with 65.84% sensitivity and 55.12% specificity. Likewise, the ROC analysis at 72hrs showed significantly higher diagnostic value of cf-mtDNA concentration with 84.25% sensitivity and 54.27% specificity (AUC, 0.8750; P = 0.0173; **Figure 2C**). However, no significant diagnostic value was observed for discriminating patients on treatment at 24hrs and 72hrs which is evident by only 57.14% sensitivity and 52.59% specificity (AUC, 0.5909; P = 0.6015; **Figure 2D**).

4.3 Discriminative analysis and diagnostic significance of relative ND1 expression levels

The concentrations of cir-mtDNA in plasma samples was further validated using SYBR Green-based RT-qPCR analysis of Ct values of mitochondrial DNA sequence ND1 relative to 12S endogenous control. This analysis showed significant difference between Ct values of ND1 between control individuals and patients at disease onset (MD, -7.457±2.030; CI, -11.56 to -3.359; p<0.0001; **Figure 3A**), at 24hrs of treatment (MD, -0.5316±0.1454; CI, -0.8281 to -0.2350; p<0.0001; **Figure 3B**), and at 72hrs of treatment (MD, -0.6572±0.1983; CI, -1.067 to -0.2479; p<0.001; **Figure 3C**). ROC analysis for relative ND1 expression levels at disease onset showed significantly higher diagnostic value with 68.70% sensitivity and 72.44% specificity (AUC, 0.9021; p<0.0001; **Figure 3D**). Likewise, ROC analysis at 24hrs of treatment showed significantly higher diagnostic value of ND1 expression (AUC, 0.8115, *P* = 0.002) with 69.47% sensitivity and 62.65% specificity, however, it was 9% lower compared to disease onset patients Almost similar diagnostic value was observed at 72hrs of treatment (AUC, 0.9083; *P* = 0.002) with 82.66% and 59.80% specificity for ND1 relative expression.

4.4 Intergroup analysis and diagnostic significance of relative ND1 expression levels

Intergroup analysis of relative expression levels of ND1 showed significantly reduced levels at 24hrs (MD, 6.926; CI, - 1.874 to 11.98; p<0.001), and at 72hrs of treatment (MD, 6.800; CI, 1.614 to 11.99; p<0.001) compared to patients at disease onset (**Figure 4A**). ROC analysis showed significantly higher diagnostic value of ND1 relative expression to discriminate patients at disease onset and 24hrs of treatment with 64.31% sensitivity and 57.75% specificity (AUC, 0.7147; *P* = 0.033; **Figure 4B**), and at 72hrs of treatment (AUC, 0.8974; *P* = 0.0007; **Figure 4C**). While, no diagnostic significance of ND1 relative expression values was observed for discriminating patients at 24hrs and at 72hrs of treatment (AUC, 0.5139; *P* = 0.89; **Figure 4D**) and represented only 51.14% sensitivity and 50.28% specificity.

4.5 Correlation analysis of cf-mtDNA concentration and relative ND1 expression

Correlation analysis of circulating cf-mtDNA concentrations showed positive relationship between control and onset (*r* = 0.17; *P* = 0.404), control and 24hrs of treatment (*r* = 0.06; *P* = 0.863; **Figure 5A & 5B**). However, negative correlation was

observed between control and 72hrs of treatment (r=-0.36; P = 0.642), onset and 24hrs of treatment (r=-0.40; P = 0.228), and onset and 72hrs of treatment (r=-0.09; P = 0.908), and 24hrs of treatment and 72hrs of treatment (r=-0.82; P = 0.181).

Furthermore, correlation matrix analysis of relative expression levels of ND1 showed significantly negative correlation between control and onset (r=-0.61; P = 0.004), control and 24hrs of treatment (r=-0.62; P = 0.022; **Figure 5C & 5D**). Although onset and 72hrs of treatment showed negative correlation, no statistical significance was achieved (r=-0.27; P = 0.395). While, correlations between other groups showed positive association including onset *vs* 24hrs of treatment (r = 0.05; P = 0.867), and 24hrs *vs* 72hrs of treatment (r = 0.30; P = 0.401).

4.6 Multiple linear regression

4.6.1 Concentration of cf-mtDNA

The disparity of cf-mtDNA values didn't show significant deviation from its linear proportion and demonstrated continuous increasing trend for control *vs* patients with stroke onset (CI, 2.259 to 2.45; P = 0.128; **Figure 6A**), and control *vs* patients at 72hrs of treatment (CI, 1.906 to 3.075; P = 0.83; **Figure 6C**). However, cf-mtDNA values for control *vs* patients at 24hrs of treatment showed significant association in linear proportion with continuous increasing trend (CI, 2.255 to 2.543; P = 0.050; **Figure 6B**). Similarity, an increasing trend and non-significant deviation of cf-mtDNA concentration values were observed for onset *vs* patients at 24hrs of treatment (CI, 7.572 to 24.19; P = 0.356; **Figure 6D**), onset *vs* at 72hrs of treatment (CI, 40.30 to 72.99; P = 0.279; **Figure 6E**), and at 24hrs *vs* 72hrs of treatment (CI, 17.01 to 78.58; P = 0.74; **Figure 6F**).

4.6.2 Relative ND1 expression levels

Although significant deviation of ND1 relative expression levels was observed between control and stroke onset patients (CI, 0.4319 to 0.8875; P = 0.039; **Figure 7A**), and control and at 24hrs of treatment patients (CI, 0.8292 to 2.550; P = 0.031; **Figure 7B**), an increasing trend was reported in their linear response. The control and at 72hrs of treated patients didn't show significant deviation from its linear proportion and

demonstrated continuous increasing trend (CI,-9.746 to 9.316; P = 0.7664; **Figure 7C**). However, a significant linear association with continuous increasing trend was reported between onset patients *vs* at 24hrs of treatment (CI, 0.2670 to 1.525; P = 0.005; **Figure 7D**), onset patients *vs* at 72hrs of treatment (CI, 35.12 to 87.97; P = 0.000; **Figure 7E**), and at 24hrs *vs* at 72hrs of treatment (CI, 7.757 to 4.86; P = 0.021; **Figure 7F**).

4.7 Correlation of NIHSS score at baseline with cf-mtDNA concentration and relative ND1 expression

The correlation analysis of baseline NIHSS score with cf-mtDNA concentration in all the enrolled study patients showed positive relationship (r = 0.6353; P = 0.0001; **Figure 8A & 8B**) which was evident with the increasing or decreasing trend of cf-mtDNA concentration values in relation to the higher or lower NIHSS score respectively. Similar observations were reported for ND1 relative expression values when correlated with the baseline NIHSS score of individual patients (r = 0.7277; P = 0.0001; **Figure 8C & 8D**).

DISCUSSION

This study assessed the ability of plasma cf-mtDNA levels to determine its role in diagnosing patients with AIS. For quantitative estimation of cf-mtDNA, we opted two separate widely used, and highly specific and sensitive tools of 1) nanodrop reading for cf-mtDNA concentration, and 2) RT-qPCR analysis for relative fold change in ND1 expression. These analyses were conducted in patients with onset of ischemic stroke and at 24hrs and at 72hrs of treatment with TPA or anti-platelet therapy. The findings of our study revealed significantly higher values of cf-mtDNA concentration as well as differences in relative fold expression of ND1 gene in AIS patients at the disease onset compared to healthy control participants. Both the assays had >64% sensitivity and >55% specificity. Although both the sensitivity and specificity were higher for ND1 expression, ROC analysis showed higher diagnostic significance of cf-mtDNA concentration estimated through nanodrop reading (AUC, 0.9808) than RT-qPCR (AUC, 0.9021). While, both the tools provide optimum outcome, quantifying cf-mtDNA

through nanodrop reading can provide more easy to use, cost-effective, and sensitive tool for diagnostic implication in patients with AIS. However, such outcome measures can be seen only when we selectively extract and quantify cf-mtDNA rather than total cfDNA (that includes both nuclear and mitochondrial DNA).

Although some of the earlier studies have reported significant association of total cfDNA quantity in patients with stroke, they failed to demonstrate individual role of cf-mtDNA and cf-nuclear DNA in discriminating AIS patients from general population [12, 7, 13]. Some of the recent studies have also demonstrated significant involvement of cf-mtDNA in distinguishing stroke with healthy population [19]. Recent studies have also demonstrated that next-generation sequencing (NGS) can provide more comprehensive tool for the quantification of mtDNA copy number [22, 23]. However, the output from NGS dataset depends on the ratio of sequencing reads of nuclear and mtDNA, but it allows analysis of thousands of available data sets which is shared by the research consortia. Although this technique enables high-sensitivity, high-throughput, and accurate assessment of mtDNA levels, a series of normalizations are required to correct for purity, counts, and batch biases [24, 25]. Moreover, NGS poses huge economic burden to the patients or their parents, hence tools presented in our study can be more widely applicable for exploring a non-invasive, simple, and cost-effective assessment with reduced burden in developing countries.

In addition, we have also explored usability of quantitative assessment of cf-mtDNA before and during the treatment of AIS patients. Intergroup analysis of patients at onset and at treatment showed significantly reduced levels of both cf-mtDNA measured by nanodrop reading (84.25% sensitivity and 54.27% specificity), and ND1 relative expression (64.31% sensitivity and 57.75% specificity) assays at 72hrs of treatment which was almost similar to the control individuals. These findings represent highly significant diagnostic value of quantifying cf-mtDNA concentration as well as ND1 expression in prognostication of the stroke patients. In accordance with our study results, a recent international, multicenter case-control study conducted on 3,498 cases of acute, first stroke from 25 countries showed buffy coat mtDNA copy number as a

robust marker of post-stroke, and determinant of related outcomes [26]. Several other studies have also demonstrated the role of cf-mtDNA in various diseases and different types of cancers [27-29].

During technical comparison, we observed that ND1 expression also provides significant difference at 24hrs, and at 72hrs of treatment (P = 0.0007); however, nanodrop quantification of cf-mtDNA didn't reveal any such difference (P = 0.6015). Furthermore, cf-mtDNA concentration by nanodrop quantification failed to demonstrate significant difference between onset and at 24hrs of treatment patients ($p>0.05$). However, RT-qPCR based quantification of cf-mtDNA through ND1 expression showed significant difference between values at onset and at 24hrs of treatment ($p<0.001$) which also predicted significant diagnostic value between the groups during ROC curve analysis (P = 0.0330). This result revealed that RT-qPCR can provide more specific and sensitive information during prognostication of patients with AIS. Furthermore, correlation as well as multiple linear regression analysis in our study showed relatively higher positive and negative predictive values, and diagnostic and prognostic significance of RT-qPCR-based ND1 expression compared to nanodrop-based cf-mtDNA concentration. Similarly, a study by Jiménez *et al* (2017) showed that mtDNA estimation can predict its applicability in differentiating severe AIS stroke patients with or without infections [30].

Comparatively lower sensitivity and specificity of cf-mtDNA quantification using nanodrop reading in our study can be explained based upon their source of release and mechanism of cell death. Cell death can be triggered either in form of apoptosis or necrosis which is turn releases cf-mtDNA in circulation. However, changes in cerebral blood-flow, ischemia to the brain parenchyma, inflammation, and neuronal cell damage all contribute to apoptosis and neurological impairment in stroke patients and ultimately affects the amount of mtDNA release [31]. Hence, there is chance of variability from patient to patient in cf-mtDNA concentration using nanodrop-based quantification. In contrast to this, ND1 is a high copy number gene in mitochondrial genome which is the initial component of the oxidative phosphorylation system. Hence,

quantifying ND1 relative expression levels or copy numbers may provide more specific information to predict cellular response in real-time [32]. Several studies support our findings for increased sensitivity and specificity of RT-qPCR-based analysis of cf-mtDNA [33-35].

Besides these findings, the type of cells involved in release of the source mtDNA must be determined focusing on the molecular mechanisms responsible for the AIS that may provide crucial insights in understanding the pathophysiology and response to the treatment. Furthermore, specifically quantifying cf-mtDNA with associated co-morbidities and patient's outcome in long-term follow-up may produce more specific response. In our study, 80% cases had anterior circulation, while the number of cases in remaining 20% was very less, so we couldn't separate the data based on anterior and posterior circulation. We also couldn't correlate our findings with the primary outcome measure after stroke using modified Rankin scale (mRS) score which can categorize independent stroke survivors from dependents. Therefore, further studies with larger sample size and prolonged follow-up in correlation with moreappropriate clinical outcome measured is required to validate cf-mtDNA quantification as precise and reproducible tool to measure AIS severity and outcome in real-time.

CONCLUSION

Quantification of cf-mtDNA in circulation using nanodrop reading or RT-qPCR-based assays may provide a simple, highly sensitive and specific, non-invasive, and affordable approach for real-time monitoring and prognostication of AIS patients at onset and during treatment. Hence, this approach may provide a widely acceptable and applicable platform at relatively lower cost and time for different clinical conditions other than AIS with further exploration.

ARTICLE HIGHLIGHTS

Research background

1. Role of circulating cell-free mitochondrial DNA (cf-mtDNA) in assessing disease status and treatment response of acute ischemic stroke (IS) patients.
2. Quantitative discrimination of IS patients from general population using cf-mtDNA.
3. Compared sensitivity and specificity of nanodrop reading and RT-qPCR tools for quantifying cf-mtDNA.

Research motivation

1. AIS results in continuously increasing rate of morbidity and mortality, and reduced quality of life worldwide.
2. Cellular apoptosis and necrosis are major events during AIS.
3. The amount of DNA present in circulation is directly proportion to the host cell death and response.

Research objectives

To validate quantitative role of cf-mtDNA in discriminating AIS patients from general population, and identifying the treatment response while comparing sensitivity and specificity of nanodrop reading and RT-qPCR tools

Research methods

1. Nanodrop reading and RT-qPCR was used to quantify cf-mtDNA in circulation.
2. Sensitivity and specificity of both the assays was measured using relative operator characteristic (ROC) curve analysis.
3. Correlation analysis of cf-mtDNA was performed with NIHSS score.

Research results

1. The findings of our study revealed significantly higher values of cf-mtDNA concentration as well as differences in relative fold expression of ND1 gene in AIS patients at the disease onset compared to healthy control participants.

2. ROC analysis showed higher diagnostic significance of cf-mtDNA concentration estimated through nanodrop reading than RT-qPCR.

3. Intergroup analysis of patients at onset and at treatment showed significantly reduced levels of both cf-mtDNA measured by nanodrop reading, and ND1 relative expression assays at 72hrs of treatment.

4. During technical comparison, we observed that ND1 expression also provides significant difference at 24hrs, and at 72hrs of treatment; however, nanodrop quantification of cf-mtDNA didn't reveal any such difference.

Research conclusions

Quantification of cf-mtDNA in circulation using nanodrop reading or RT-qPCR-based assays may provide a simple, highly sensitive and specific, non-invasive, and affordable approach for real-time monitoring and prognostication of AIS patients at onset and during treatment.

Research perspectives

This approach may provide a widely acceptable and applicable platform at relatively lower cost and time for different clinical conditions other than AIS with further exploration.

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