The value of serial plasma nuclear and mitochondrial DNA levels in adult community-acquired bacterial meningitis

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Summary

Background: Increased levels of plasma nuclear and mitochondrial DNA have been reported in critically ill patients. We tested the hypothesis that plasma nuclear and mitochondrial DNA are substantially increased in acute bacterial meningitis and decrease after antimicrobial therapy, and that plasma nuclear and mitochondrial DNA levels can predict treatment outcomes.
Methods: We examined serial plasma nuclear and mitochondrial DNA levels in 22 adult community-acquired bacterial meningitis (ACABM) patients. The plasma nuclear and mitochondrial DNA levels were also evaluated in 11 aseptic meningitis patients and 22 volunteer subjects during the study period.
Results: All of the both bacterial and aseptic meningitis groups had a higher plasma DNA levels on admission as compared with those of volunteer groups. Levels of plasma nuclear and mitochondrial DNA in ACABM cases were significantly increased initially and substantially decreased thereafter. Both plasma nuclear DNA and plasma mitochondrial DNA levels at presentation are significantly negative correlate with modified Barthel Index (average) ($r = -0.639$, $P = 0.004$ and $r = -0.551$, $P = 0.018$) at 3 months after discharge (average), respectively, in this study. Both higher plasma nuclear (cutoff value of >169 ng/ml) and mitochondrial DNA levels (cutoff value of >58.9 ng/ml) at presentation were associated with poor outcome in ACABM patients.
Conclusions: Based on our results, the higher plasma DNA levels were associated with a poorer outcome. Therefore, we look forward to more prospective multicenter investigations specifically to confirm the predictive value of plasma DNA levels in outcome prediction.

Introduction

Despite the advent of new antimicrobial drugs, mortality and morbidity from adult community-acquired bacterial meningitis (ACABM) remains high.[1] The prognosis of patients is important in risk stratification and for the efficient use of hospital resources. Predicting the outcome of patients in the intensive care environment is of particular significance, to ensure that resources are used appropriately. Unfavorable neurologic outcomes may not be the result of treatment with inappropriate antimicrobial agents, since cerebrospinal fluid (CSF) cultures are sterile 24–48 h after the start of antimicrobial therapy.[2]

Relatively little is known about free circulating DNA, but several studies have established that baseline levels are present in normal, healthy

populations, albeit at very low levels.[3,4] The early and high concentrations of plasma DNA observed in critically ill patients suggest that extracellular DNA may originate from damaged tissues, such as necrosis.[5] Impaired clearance is another possible reason for the increase in cell-free DNA. However, after critically ill conditions occur, organs responsible for the elimination might be damaged as a consequence of ongoing systemic inflammation.[6]

Increases in plasma nuclear and mitochondrial DNA occur in a variety of critical conditions associated with cell death, including cancer,[3] trauma[7] and stroke.[8] These studies involved only one blood sample taken from each patient, and it was evident that there was considerable variation among patients. Such variability may be explained by rapid changes in the balance of production and clearance of DNA in the plasma. If such variation followed a standard pattern and temporal relationship, it would reduce variance and improve our ability to predict the prognoses.

To our knowledge, little is known about the time course of changes in plasma nuclear and mitochondrial DNA concentration in bacterial meningitis patients and more accurate information about changes may be gained by taking serial rather than single blood samples during antimicrobial therapy. As ACABM involve cell death and disruption of the blood–brain barrier,[9] we hypothesized that DNA would be liberated early into the plasma after the onset of meningitis and might be useful for assessing disease severity and for predicting therapeutic outcome. Through this prospectively designed study, we aimed to report on the relationship between serial nuclear and mitochondrial DNA levels in plasma and therapeutic outcome in ACABM patients.

Patients and methods

This prospective study on the time course of plasma nuclear and mitochondrial DNA levels in ACABM patients was conducted over a 1-year period (July 2008–June 2009). During this period, 22 adult (≥16-years old) ACABM patients at the Chang Gung Memorial Hospital (CGMH)-Kaohsiung were enrolled. CGMH-Kaohsiung, a 2482-bed acute-care teaching hospital, is the largest medical center in southern Taiwan that provides both primary and tertiary referral care.

In this study, the diagnostic criteria for ACABM included a positive culture result of CSF in patients with clinical presentations of bacterial meningitis, with at least one of the following parameters of purulent CSF inflammation: (i) leucocytosis with leucocyte counts $>250 \times 10^6$/l and predominant polymorphonuclear (PMN) cells; (ii) CSF glucose ratio (CSF glucose/blood glucose) <0.4; or (iii) CSF glucose <2.5 mmol/l if without blood glucose.[10,11] In patients with negative culture results, a diagnosis of acute bacterial meningitis is based on compatible clinical features and pleocytosis of at least 100×10^6 PMN/l.[10] Bacterial meningitis was characterized as either being nosocomial or community acquired.[1,12] 'community-acquired bacterial meningitis' was defined as bacterial meningitis contracted outside a hospital environment. The study protocol was approved by Chang Gung Memorial Hospital's Institutional Review Committee on Human Research.

For comparison, both 11 aseptic meningitis patients and 22 volunteers with or without cardiovascular risk were included as controls. The diagnostic criteria for aseptic meningitis included clinical evidence of acute meningitis such as fever, headache or other signs of meningeal irritation, absence of any microorganism on Gram stain of CSF, negative routine bacterial culture of CSF in the absence of antibiotic treatment before obtaining the first CSF sample, and CSF parameters of leucocytosis with leucocyte counts $>15 \times 10^6$/l if the patient was 2 months of age or older. A small and variable increase in protein and glucose content of the CSF is normal.[13] The plasma nuclear and mitochondrial DNA levels were also compared with the normal value of our laboratory. The normal values were obtained from 22 volunteers with or without cardiovascular risks whose ages ranged between 47 and 63 years (Table 1).

In every patient, 3 ml of peripheral venous blood was collected into EDTA-containing tubes at presentation (Day 1). Follow-up blood samples studies were fixed for Days 7, and Days 14 after admission. Procedural details had been described previously.[14,15] To ensure cell-free plasma collection, samples were initially centrifuged for 10 min at 3000 r.p.m., followed by separation into a 1.5 ml clear polypropylene tube taking care not to disturb the buffy coat layer. The newly separated aliquot was centrifuged for a further 10 min at 10 000 r.p.m., after which the upper portion of plasma was removed by a Pasteur pipette (~500 μl), was placed into a further clear tube and was frozen at −20°C prior to extraction. DNA was extracted from plasma samples by use of a QIAamp Blood Kit (Qiagen) according to the 'blood and body fluid protocol' recommended by the manufacturer. We used 200 μl of the plasma sample, per column, for DNA extraction. The exact amounts used were documented to enable calculation of target DNA concentration.

Table 1 Baseline characteristics of the bacterial meningitis, aseptic meningitis and control groups

	Bacterial meningitis $n = 22$	Aseptic meningitis $n = 11$	Control group[a] $n = 22$
Age (year) (mean ± SD)	56.0 ± 17.2	34.5 ± 16.0	57.4 ± 4.7
Male/Female	11/11	4/7	14/8
Underlying diseases			
Diabetes mellitus	10	1	5
Hypertension	9	1	15
Liver diseases/alcoholism	6	1	0
Current smoking	3	2	0
Chronic otitis media	1	0	0
Hyperlipidemia	1	0	15
End stage renal disease	1	0	0
Glasgow coma score	11.0 ± 4.1	15	15
Causative pathogens			
Gram-negative bacilli[b]	7	—	—
Streptococcal species[c]	5	—	—
Staphylococcal species[d]	4	—	—
Other pathogens or negative	6	—	—
Laboratory data at the time of admission			
Mean (SD) Glucose/blood glucose ratio	0.46 (0.15)	0.62 (0.17)	—
Mean (SD) Total protein (g/l)	2.83 (1.53)	1.24 (0.23)	—
Mean (SD) Lactate (mmol/l)	7.56 (4.30)	3.06 (0.72)	—
Mean (SD) white cell count ($\times 10^6$/l)	1639 (546)	150 (61)	—
Median (IQR) plasma nuclear DNA (ng/ml)	100 (30–210)	34 (13–49)	22 (15–32)
Median (IQR) plasma mitochondrial DNA (ng/ml)	22 (15–75)	17 (9–20)	10 (6–16)

SD: Standard deviation; IQR: interquartile range.
[a]Patients follow-up at outpatient clinic.
[b]The causative pathogens included *Klebsiella pneumoniae*[6] and *Escherichia coli.*[1]
[c]The causative pathogens included and *Streptococcus pneumoniae*[3] and *Viridians streptococci.*[2]
[d]*Staphylococcus aureus.*[4]

Plasma DNA was measured by a real-time quantitative PCR assay (Roche Lightcycler; Roche, Lewes, UK) for the β-globin gene and ND2 gene.[14,15] On each run, a standard curve was repeated as well as with the inclusion of a positive genomic DNA control and a negative (de-ionized water) control. The β-globin gene is present in all nucleated cells of the body while ND2 gene is specific mitochondrial DNA. The β-globin PCR system consists of the amplification primers β-globin-354F (5′-GTG CAC CTG ACT CCT GAG GAG A-3′) and β-globin-455R (5′-CCT TGA TAC CAA CCT GCC CAG-3′). The PCR probe contained a 3′-blocking phosphate group to prevent probe extension during PCR. Expression of mtDNA were measured by quantitative RT-PCR based on continuous measurements of Syber green fluorescent dye that binds to double-stranded DNA generated during PCR and a specific primer pair for ND2 (forward: 5′-CAC AGA AGC TGC CAT CAA GTA-3′; reverse: 5′-CCG GAG AGT ATA TTG TTG AAG AG-3′). The imprecision of this system had been reported previously, with a CV of the threshold cycle of 1.1%. The expression of

quantitative results as ng/ml was as described previously.[14,15]

Therapeutic outcomes of >3 months after discharge were evaluated by using a modified Barthel Index (MBI).[16] For the purposes of analysis, a score below 12 was defined as a poor outcome, and 12 or more as good. Death was included in the poor outcome group.

Data were expressed as mean ± SD or median (interquartile range). Categorical variables were compared using Chi-square test or Fisher's exact test. Levels of plasma nuclear and mitochondrial DNA were logarithmically transformed to improve normality. Univariate analyses were compared using Student's *t*-test. Continuous variables among three groups (bacterial meningitis, aseptic meningitis and volunteers) were compared using one-way ANOVA, followed by *post hoc* multiple comparison procedure. Repeated measures of ANOVA were used for comparison of plasma nuclear and mitochondrial DNA among three intervals (at presentation: Days 1, 7 and 14). We used Scheffe multiple comparison to analyze the intraindividual

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course of parameters over time and to compare parameters of different groups (good and poor outcome) of bacterial meningitis patients. Correlation analysis was used to explore the relationship between Glasgow coma score (GCS) on admission and the score of MBI at 3 months after discharge (average) and variables such plasma nuclear and mitochondrial DNA levels of bacterial meningitis group on admission. Further, stepwise logistic regression was used to evaluate the relationship between significant variables and therapeutic outcomes, with adjustments made for other potential confounding factors. Variables with a zero cell count in a two-by-two table were eliminated from logistic analysis, while only variables with a strong association with fatality rate ($P < 0.05$) were included in the final model. Receiver operating characteristic (ROC) curves was generated for plasma nuclear and mitochondrial DNA levels on admission. The areas under the ROC curves were calculated for each parameter and compared. All of the statistical analyses were conducted using the SAS software package, version 9.1 (2002, SAS Statistical Institute, Cary, NC, USA).

Results

Baseline characteristics of the study patients

The baseline characteristics of the 22 adult ACABM cases, 11 aseptic meningitis patients and 22 volunteer subjects are listed in Table 1. Of the ACABM cases, 18 had one or more underlying disease. Regarding causative pathogens, Gram-negative bacilli were the most prevalent, followed by *Streptococcus* and *Staphylococcus* species.

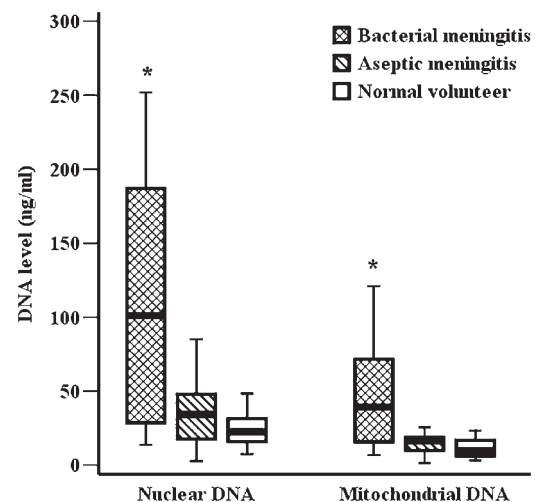### Time course of plasma nuclear and mitochondrial DNA levels

The median value of plasma nuclear and mitochondrial DNA levels on admission in the bacterial meningitis, aseptic meningitis and volunteer groups are shown as a box-plot in Figure 1. Levels of plasma nuclear and mitochondrial DNA levels on admission among the bacterial, aseptic meningitis and volunteer groups revealed the following significant findings: plasma nuclear DNA levels ($P = 0.002$) and plasma mitochondrial DNA levels ($P = 0.002$) (Figure 1). The plasma nuclear DNA levels were significantly higher than volunteers groups from at presentation (Day 1) to Day 14 (Day 1: $P = 0.0001$; Day 7: $P = 0.0001$; Day 14: $P = 0.016$, respectively) (Figure 2A). Furthermore, the plasma mitochondrial DNA levels were also significantly higher than

volunteers groups from at presentation (Day 1) to Day 14 (Day 1: $P = 0.0001$; Day 7: $P = 0.0001$; Day 14: $P = 0.016$, respectively) (Figure 2B).
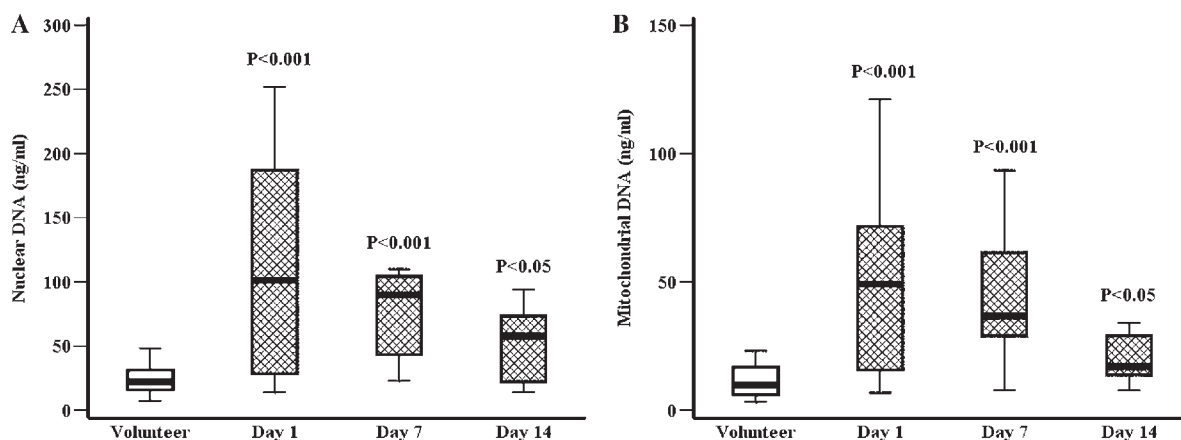
The time course of plasma nuclear and mitochondrial DNA levels in ACABM patients who had good and poor outcomes is listed in Table 2. The levels of plasma nuclear and mitochondrial DNA levels in ACABM cases were significantly increased initially and substantially decreased thereafter (Figure 2A and B). The plasma nuclear DNA levels at presentation (Day 1) were significantly higher in the poor outcome group (median = 211 ng/ml) than in the good outcome group (median = 60 ng/ml) at presentation ($P = 0.023$), and plasma mitochondrial DNA levels at presentation were significantly higher in the poor outcome group (median = 86 ng/ml) than in the good outcome group (median = 20 ng/ml) ($P = 0.015$). The difference of both plasma nuclear and mitochondrial DNA levels between the two outcome groups was no longer significant from Days 7 to 14 after ACABM. Moreover, repeated measures of ANOVA with Scheffe's multiple comparison demonstrated that both plasma nuclear and mitochondrial DNA levels between the two outcome groups at three different time points (at presentation: Days 1, 7 and 14) were significantly different ($P = 0.002$ and $P = 0.002$).

Effect of plasma nuclear and mitochondrial DNA levels on GCS score in ACABM patients

Correlation analysis was used to test the influence of age, plasma nuclear (ng/ml) and mitochondrial DNA

Figure 1. The median values of plasma nuclear and mitochondrial DNA levels among bacterial, aseptic meningitis and volunteers groups at the time of admission. *$P = 0.002$, bacterial meningitis, aseptic meningitis and volunteers groups.

Figure 2. The serial median values of serial plasma nuclear (**A**) and mitochondrial DNA (**B**) levels in patients with bacterial meningitis. Plasma nuclear NDA level: bacterial meningitis vs. volunteers groups (at presentation Day 1: $P < 0.001$; Day 7: $P < 0.001$; Day 14: $P < 0.05$). Plasma mitochondrial DNA levels: bacterial meningitis vs. volunteers groups (at presentation Day 1: $P < 0.001$; Day 7: $P < 0.001$; Day 14: $P < 0.05$)

Table 2 Time course of plasma nuclear and mitochondrial DNA in community-acquired bacterial meningitis patients who had good and poor outcomes

	Day 1		Day 7		Day 14	
	Good outcome	Poor outcome	Good outcome	Poor outcome	Good outcome	Poor outcome
Median (IQR) plasma nuclear DNA (×ng/ml)[a]	60* (21–131)	211 (184–282)	41 (25–110)	101 (67–396)	39 (17–63)	69 (51–389)
Median (IQR) plasma Mitochondrial DNA (×ng/ml)[a]	20* (11–49)	86 (38–121)	15 (13–94)	34 (31–48)	10 (10–39)	27 (18–52)

IQR: interquartile range; *$P < 0.05$.
[a]Levels of plasma nuclear and mitochondrial DNA were logarithmically transformed to improve normality, and repeated measures of ANOVA were used to compare plasma nuclear and mitochondrial DNA levels at three different time points [week one (Day1), week 2 (Day 7) and week 3 (Day 14)]. Scheffe's multiple comparison was used to analyze the intra-individual course of parameters over time and to compare parameters of two different groups of bacterial meningitis patients (good and poor outcome).

levels (ng/ml) at presentation on GCS at presentation (average). The statistical results (correlation coefficient, P-value) were as follows: age ($r = -0.531$, $P = 0.028$), plasma nuclear DNA level ($r = -0.832$, $P < 0.001$) and plasma mitochondrial DNA levels ($r = -0.298$, $P = 0.28$). Furthermore, both plasma nuclear DNA and plasma mitochondrial DNA levels on admission are positively correlated with age (average) ($r = 0.374$, $P = 0.127$ and $r = 0.092$, $P = 0.718$), respectively, in this study.

Outcome and prognostic factors

The potential prognostic factors of the 22 ACABM patients at the 3-month end-point are listed in Table 3. Statistical analysis of the clinical manifestations, laboratory data and neuro-imaging studies between the good and poor outcome groups revealed the following significant findings: diabetes mellitus (DM) as the underlying diseases ($P = 0.0001$), GCS on admission ($P = 0.037$), plasma nuclear DNA level ($P = 0.023$) and plasma mitochondrial DNA levels ($P = 0.015$). The variables used in the logistic regression included GCS on admission, plasma nuclear DNA level and plasma mitochondrial DNA levels. After analysis of the aforementioned variables, only plasma mitochondrial DNA levels at the time of admission ($P = 0.039$, OR = 0.0001, 95% CI = 0.0001–0.171) was independently associated with outcome. Both plasma nuclear DNA and plasma mitochondrial DNA levels at presentation are significantly negatively correlated with MBI (average) at 3 months after discharge (average) ($r = -0.639$, $P = 0.004$ and $r = -0.551$, $P = 0.018$), respectively, in this study. Furthermore, the area under the ROC curve for

Table 3 Prognostic factors of patients with community-acquired bacterial meningitis

	Poor outcome $n=7$	Good outcome $n=15$	OR	95% CI	*P*-value
Mean (SD) age at meningitis (year)	60.3 (15.4)	54.0 (18.2)			0.439
Gender					
Male	5	6	0.267	0.038–1.852	0.361
Female	2	9			
Underlying diseases					
Liver cirrhosis/alcoholism	4	2	0.115	0.014–0.953	0.054
Diabetes mellitus	7	3	3.33	1.293–8.591	0.001
Clinical feature					
Fever	5	11	1.1	0.149–8.125	1.0
Headache	5	10	0.8	0.113–5.679	1.0
Disturbed consciousness	5	7	0.350	0.051–2.407	0.381
Seizure	1	1	0.429	0.023–8.043	1.0
Hearing impairment	0	2	0.65	0.471–0.897	1.0
Septic shock	3	1	0.095	0.008–1.185	0.077
Metastatic septic abscesses					
Neuroimaging findings					
Hydrocephalus	3	2	0.205	0.025–1.694	0.274
Meningeal enhancement	2	5	1.25	0.176–8.874	1.0
Mean (SD) GCS at the time of admission	8.2 (4.0)	12.5 (3.5)			0.037
Laboratory data at the time of admission[a]					
Mean (SD) CSF glucose/blood glucose ratio	0.46 (0.21)	0.46 (0.13)			0.898
Mean (SD) CSF total protein (g/l)	3.56 (0.79)	2.02 (0.54)			0.630
Mean (SD) CSF lactate (mmol/l)	10.85 (2.29)	7.88 (1.64)			0.237
Mean (SD) CSF white cell count ($\times 10^6$/l)	9.79 (4.26)	7.29 (5.46)			0.913
Median (IQR) plasma nuclear DNA	211 (184–282)	60 (21–131)			0.023
Median (IQR) plasma mitochondrial DNA	86 (38–121)	20 (11–49)			0.015

GCS: Glasgow coma score; OR: odds ratio; CI: confidence interval; SD: standard deviation; IQR: interquartile range.
[a]Independent *t*- test, two-side, data were logarithmically transformed to improve normality.

plasma nuclear and mitochondrial DNA levels on admission were 0.862 ($P=0.021$, 95% CI $=0.68$–1.04) and 0.831 ($P=0.034$, 95% CI $=0.60$–1.06), respectively. The cutoff value of plasma nuclear and mitochondrial DNA levels on admission for poor outcome was 169 ng/ml (sensitivity 100% and specificity 85%) and 58.9 ng/ml (sensitivity 80% and specificity 85%), respectively.

Discussion

To our knowledge, this is the first study to show serial circulating plasma and mitochondrial DNA concentrations, assessed by measuring the β-globin gene and ND2 gene concentrations by real-time PCR, during acute phase of ACABM. We also confirmed our hypothesis that plasma nuclear and mitochondrial DNA are substantially increased in acute bacterial meningitis and decrease after antimicrobial therapy, and that plasma nuclear and mitochondrial DNA levels can predict treatment outcomes.

An increase in plasma DNA concentration may therefore occur either due to increased liberation from cells following cell death (e.g. necrosis or apoptosis) or to a decrease in clearance efficiency. In addition, the clearance mechanism of DNA from the circulation is poorly understood, although experimental studies using animals have produced evidence suggesting that the liver and the kidneys are prime candidates for its removal.[17]

In the present study, we examined the time course of plasma nuclear and mitochondrial DNA levels in ACABM patients and produced four major findings. First, all of the meningitis patients investigated had a positive result on admission and the levels of plasma nuclear and mitochondrial DNA levels in the bacterial meningitis group was significantly higher than those of the aseptic meningitis and volunteers group. Second, the plasma nuclear and mitochondrial DNA levels in the ACABM cases was significantly increased and then substantially decreased thereafter. Third, mean age at infection and plasma nuclear DNA levels at presentation were significantly negative correlate with GCS at

presentation (average). Fourth, both plasma nuclear DNA and plasma mitochondrial DNA levels at presentation are significantly negative correlate with MBI (average) at 3 months after discharge (average), respectively, in this study. Finally, both higher plasma nuclear (cutoff value of >169 ng/ml) and mitochondrial DNA levels (cutoff value of >58.9 ng/ml) at presentation was associated with poor outcome in ACABM patients.

Although our study demonstrated higher plasma nuclear and mitochondrial DNA levels on admission was associated with poor outcome in ACABM patients. There are two main limitations to this study. First, impaired clearance is another possible reason for increase in cell-free DNA,[18] and after meningitis, organs responsible for elimination might be damaged as a consequence of ongoing systemic inflammation and presence of hemodynamic crisis (e.g. septic shock). Second, the levels of the plasma nuclear and mitochondrial DNA may be influence by age and underlying conditions.[19] Our study also demonstrated that both plasma nuclear DNA and plasma mitochondrial DNA levels on admission are positive correlate with age (average) of ACABM patients. Although the sample size was not large, the numbers of variables considered for multiple logistic regression analysis was small. Furthermore, based on stepwise procedures, only three variables were selected as the important variables predicting outcome. Nonetheless, the maximum likelihood estimates of the coefficients were valid in the analysis.

Based on our results, the higher plasma DNA levels were associated with a poorer outcome. Therefore, we look forward to more prospective multicenter investigations specifically confirm the predictive value of plasma DNA levels in outcome prediction.

Funding

Conflict of interest: None declared.

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