Metabolomic profiling of cerebrospinal fluid reveals metabolite biomarkers in Tick-borne encephalitis Patient

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Abstract

Tick-borne encephalitis virus (TBEV) can cause life-threatening CNS infection. Changes in cerebrospinal fluid (CSF) metabolites may reflect critical aspects of host responses and end-organ damage in neuro infection and neuroinflammation. In this study, we applied an untargeted metabolomics screen of CSF samples to investigate the metabolites profile and explore biomarkers for TBEV infection. By analyzing CSF samples from 77 patients with TBEV infection and 23 without TBEV infection, tryptophan metabolism and Citrate cycle were found to be the top important metabolic pathways in differentiating the control and case groups; acetoacetate, 5'-deoxy-5'-(methylthio)-adenosine, 3-methyl-2-oxobutanoic acid,etc. were identified to be metabolic biomarkers(| log 2 FC|>1,VIP>1,FDR<0.05)in CSF and clearly separated the TBEV infection from the non-infected samples. Moreover, four metabolites were identified to be associated with fatal outcome, including kynurenic acid, 5-hydroxyindole-3-acetic acid, DL-tryptophan, indole-3-acrylic acid, demonstrating the potential predictive biomarkers for severe TBEV infection. This study explored the metabolic profile of TBEV infection both in CSF samples and identified candidate biomarkers for TBEV infection, which might be useful in target screening for differential diagnosis and therapeutic inter-vention.

Introduction

Tick-borne encephalitis virus (TBEV) belongs to *Flavivirus genus* ,*Flaviviridae family* . The virus is a minus- strand RNA virus with a spherical particle and envelope protein on its surface.

TBEV is transmitted primarily through the bite of the infected tick, the peak period of tick activity is from April to November each year, so TBE is generally popular in spring to autumn [1]. After tick biting, the virus can replicate under the skin of the patient. Dendritic epithelial cells in the skin may be the first cells involved in virus replication, and then the dendritic epithelial cells transport the virus through the lymphatic system to the local lymph nodes for replication, which can produce viremia after transmission. During this period, the virus will gradually infect the extra neurologic tissues (liver, spleen, bone marrow), and when the infection continues to worsen, the virus can cross the blood-brain barrier and reach the brain, resulting in central nervous system infection [2]. At this stage, patients may develop encephalitis, meningitis, meningomyelitis, cognitive dysfunction, limb spasms and seizures. Some patients do not fully recover after TBE disease, which may lead to long-term neurological sequelae such as hemiplegia.

Current diagnostics for TBEV infection include serology and qPCR[3,4], qPCR has a high specificity (>95%) and sensitivity (80–95%), but viral load does not correlate well with TBE outcome. Its usefulness may also be limited at early stages of disease, as PCR turns negative within about 1–3 weeks of detectable infection. As for serology method, antibodies are not detectable within two weeks after acute infection and can persist in humans after initial infection,so exploring novel biomarkers is in a demand for TBEV infection identification.

The metabolomic approaches are capable of noticing subtle differences and may hold the key to discover biomarkers for distinguishing among specific pathogen infection,[5-7],there are some reports about metabolomics and lipidomic of TBE serum as well [8,9], however, no detailed studies about TBE metabolic studies on cerebrospinal fluid(CSF) samples have been reported yet. TBEV infection can induce severe encephalitis, studying the metabolites changes in CSF after TBEV infection could help to reveal the molecular mechanism of TBEV infection in the central nervous system and provide potential targets for the diagnosis, prognosis, monitoring or treatment of TBE.

In this study, we conducted an untargeted metabolomics analysis of CSF from TBE patients using LC-MS technology, the differential metabolites and the candidate biomarkers in CSF were screened, and the differential metabolites associated with fetal cases in CSF were also analyzed.

Material and methods:

Sample collection

TBE patients was diagnosed by detection of anti-TBEV IgM antibodies (or the titer of IgG in the convalescence sera increased quadruple than that in the acute phase) by ELISA.A total of 100 clinical samples were collected in this study, including 77 CSF samples from TBE patients, 23 CSF samples from non-TBE participants. Among the patients providing CSF samples, 5 patients eventually died within 60 days after TBEV infection.

The 23 non-TBE participants for CSF samples included 6 patients with brain tumor, 15 with cerebral apoplexy,and 2 with bacterial meningitis.

CSF samples of TBE patients were from the Mudanjiang forest hospital in 2011-2013, the non-TBE samples were from the PLA general hospital in 2020-2023, all of them were obtained during initial routine lumbar puncture. Written informed consent has been obtained from all participants. This study has been approved by the Ethical Board of the academy of military medical science (No.AP/SC-08/02.128).**All of the samples were** stored in a refrigerator at -80°C without repeated freeze-thaw.

Metabolite extraction.

100 μL of the CSF samples were extracted by adding 400 μL of methanol extraction solution; after vortexing for 20 s, the samples were placed in ice water for 5 min and then centrifuged at 15,000 g for 20 min at 4°C; the supernatant was diluted with mass spectrometry-grade water until the methanol content was 53%; The samples were then centrifuged at 15,000 g at 4°C for 15 min. The supernatant was collected for further analysis.

Metabolomics test by liquid chromatography-mass spectrometry (LC-MS)

The collected supernatant was separated using a Vanquish ultra-high liquid chromatography(UHPLC, Thermo Fisher, Germany) and a mass spectrometer(Q Exactive HF-X,Thermo Fisher,Germany),with a Hypesil Gold column (100 x 2.1 mm, 1.9 μm) temperature of 40°C, flow rate of 0.2mL/min. The mobile phases can be divided into positive and negative ionic modes, with the positive ionic mode consisted of 0.1% formic acid (phase A) and methanol (phase B), and the negative ionic mode consisted of 5 mM ammonium acetate at pH 9.0 (phase A) and methanol (phase B). The gradient elution procedure was as follows: 0 to 1.5 min, 2% B;1.5 to 3 min, B was increased linearly from 2 to 85%; 3 to 9 min, B was gradually increased to 100%.

To monitor the stability of the system in real time, a pooled sample generated by taking a small volume of each experimental sample to serve as a quality control (QC) sample that was run multiple times throughout the experiment, extracted water samples served as blanks. a mixture of internal standards was also spiked into every sample to aid chromatographic peak alignment and instrument stability monitoring.

The electrospray ionization (ESI) positive and negative ion modes were used for mass spectrometer (MS) detection. The scanning range was m/z100-1500; the ESI source parameters were set as follows: Spray Voltage 3.5kV for positive mode, Spray Voltage 3.2kV for negative mode, Sheath gas flow rate 40 psi, Auxiliary gas

flow rate 10L/min, Capillary Temp 320°C, S-lens RF level 40, Auxiliary gas heater temp 350°C.Positive and negative ion modes were data-dependent scanned separately..

The raw data were imported into Compound Discoverer 3.3 (Thermo Fisher Scientific) software for data pre-processing, peak finding/alignment, and peak annotation,and the metabolites were identified .

Statistical analysis

In this study, normality tests were performed on the TBE and control groups using SPSS version 27.0 (IBM, New York, USA) software. The independent samples t-test was used for variables that conformed to a normal distribution and Homogeneity of variance, and the Mann-Whitney U-test for variables that were not normally distributed, as a means of determining whether there was a significant difference in the concentration of each metabolite between the TBE group and the control group. p-values were two-tailed and $p<0.05$ was considered statistically significant.

False Discovery Rate (FDR) is the percentage of all findings where errors occur and is commonly used to correct p-values in multiple hypothesis tests. The p values for all metabolites in each comparison were adjusted by the spurious discovery rate method, and the false discovery rate (FDR) < 0.05 was considered statistically significant.

In addition, fold change (FC) thresholds were also used as an indicator to differentiate between groups, with the magnitude of the FC value reflecting the extent to which the metabolites differed between the two groups of samples. Metabolites with FC>1.5 or FC<0.66 (|FC>1.5)were considered to be different.

SIMCA-P 14.1 software package (V14.1; Sartorius Stedim Data Analytics AB, Umea, Sweden) was used for multidimensional statistical analysis, including principal-component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) [37]. Using OPLS-DA analysis, a VIP value can be derived for each metabolite, with variable importance in prediction (VIP)> 1 contributing more to the distinction between the two groups.

The PCA maps, volcano maps, and cluster maps were generated with the R program. KEGG enrichment analysis was utilized to determine the metabolic pathways[4,10].

Single-dimensional statistical analysis was performed using SPSS Statistics version 27.0 (IBM, New York, USA). Student's t test and variation multiple analyses were used for comparison of the raw data. A P value or False Discovery Rate (FDR) of 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curves were plotted and taken a value between 0.5 and 1, with closer to 1.0 indicating better diagnostic performance.

Results:

Metabolomic Profiling of cerebrospinal fluid from Tick-borne encephalitis Patient

We procured a cohort of patients containing 77 TBE, and 23 non- TBE patient as controls (including 6 brain tumor patient, 15 cerebral apoplexy patients, 2 bacterial meningitis patients) for analysis of metabolomic profiling in CSF, the age and the sex composition were all comparable between the case and control groups. (Table S1)

Through ultra performance liquid chromatog-raphy/tandem mass spectrometry (UPLC-MS/MS) untargeted metabolomics analysis, we identified a total of 705 cationic compounds and 284 anionic compounds (including 95 drugs and their metabolites) detected from CSF samples. Drugs and their metabolites are not used in the following analysis.

For metabolomic analysis, both hydrophilic and hydrophobic molecules were analyzed using positive and negative ionization to cover various endogenous biochemical classes. The orthogonal partial least-squares discrimination analysis (OPLS-DA) was used to assess nonorthogonal and orthogonal variables, and the key model parameters R^2Y and Q^2 were employed to evaluate the fitness and prediction ability of the model, respectively. As shown in Figure 1A, the metabolomic data of CSF from TBE patients were well resolved from

non-TBE individuals,of which the key parameters were R^2 Y =0.963 and Q^2 = 0.885,indicating moderate fitness and predictability. In addition, the results of the 200-permutation test in the OPLS-DA model also proved that the model is robust and not overfitted (Figure 1B). These analyses showed that most of the samples were correctly categorized into TBE and control groups.

Metabolomic Changes in CSF of TBE patients and metabolic pathways.

We analyzed the metabolites that underwent significant change using 20 non-infectious CSF as a control group(Con) and 72 TBE -infected CSF as TBE group (TBE). False Discovery Rate (FDR) is the percentage of all findings where errors occur and is commonly used to correct p-values in multiple hypothesis tests. The p values for all metabolites in each comparison were adjusted by the spurious discovery rate method, and the false discovery rate (FDR) < 0.05 was considered statistically significant.And fold change (FC) for each metabolite was calculated, metabolites with FC>1.5 or FC<0.66 (|FC>1.5)were considered to be different.

It was found that 162 metabolites were significantly changed [TBE vs. Con, |FC|>1.5, FDR<0.05] in TBE group. The details of the differential metabolites are shown in Table S2. The volcano plot was used to show the different metabolites, with each point in the volcano plot representing a metabolite. The volcano plot revealed 54 increased metabolites and 108 decreased metabolites in TBE group (figure 1C). The heatmap (Figure 1D) was used to show the hierarchical clustering of the metabolite data.

To investigate the role of the differential metabolites during TBEV infection, we searched the metabolism pathways associated with the 162 differential metabolites. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Metabolome Database were used to search the corresponding pathway database and further identify the corresponding pathway database related to the changes in metabolites. The results of the metabolic pathways analysis are shown by a bubble plot (Figure 1E), the panel of differential 162 metabolites was enriched into 29 pathways. The significantly altered five pathways ($p < 0.05$) were valine, leucine and isoleucine biosynthesis citrate cycle (TCA cycle)、tryptophan metabolism、tyrosine metabolism and purine metabolism.

It was found that a total of 20 metabolites contributed to the above five metabolism pathways by KEGG annotation, and 11 metabolites were involved in amino acid metabolism,demonstrating the most changed metabolism pathway in the central nervous system during TBE infection (Table S3).

Tryptophan(Trp)is an important amino acid that plays an indisputable role in several physiological processes, including immune responses and neurological function[4].The differential metabolites matched on the tryptophan metabolism pathway are Ltryptophan, 2-(formylamino) benzoic acid, 5-hydroxytryptophan and indole-3-acetic acid, all of them were decreased in the CSF of TBE patients, indicating that tryptophan metabolism was significantly disturbed during TBE infection, which might be related to the inflammation and nerve damage in patients after TBEV infection.

The five changed metabolites (adenosine、deoxyadenosine、xanthosine、inosine、2,6-dihydroxypurine) involved in purine metabolism were all decreased in TBE patients, and the three changed metabolites (cis-aconitic acid、isocitric acid、alpha-ketoglutaric acid) involved in citrate cycle (TCA cycle) were increased in TBE patients, which indicated the higher energy consumption in TBE patients.

Selection and validation of TBE biomarkers in CSF

This study aimed to identify a set of biomarkers for the TBE infection in CSF. In general, variable importance in prediction (VIP) was used to reflect the importance of the variables in the OPLS-DA model. Combining the p-value of the significance test and the FC-value, a total of 48 differential metabolites with VIP>1, FDR<0.05, $|\log_2 FC|>1$ were screened out as candidate biomarkers to distinguish TBE group from control group, with 8 metabolites significantly increased and 40 metabolites significantly decreased. The heatmap of the 48 candidate biomarkers was shown in Fig 2A and the 48 candidate biomarkers in CSF were concluded in Table 1.

Then ROC analysis was performed to select metabolites that could be applied to classified TBE patients

from non-TBE individuals, there are 32 metabolites showed AUC>0.8 and 20 candidate biomarkers showed AUC>0.9, suggesting the 20 metabolites with AUC>0.9 can be used as biomarkers to well distinguish TBE patients from non-TBE individuals. The $|\log_2 FC|$ and the AUC value were shown in Figure 2B, the concentration of the 20 biomarkers were compared (TBE vs Control) and demonstrated in in Figure 2C. Among the 20 biomarkers(AUC>0.9), 12 biomarkers were significantly decreased and 8 biomarkers were significantly increased, and it is noteworthy that the biomarkers with the top two AUC were acetoacetate and 5'-deoxy-5'-(methylthio)- adenosine**,** which were decreased significantly, with the $|\log_2 FC|>3$ and AUC>0.95, and would be potentially useful for TBE identification.

Acetoacetate(FDR<0.05, $\log_2 FC$=-3.62, AUC=0.983), a naturally occurring metabolite produced by the liver, is used by monocytes/macrophages as an alternative fuel to alleviate lactic acidosis-induced pseudo-starvation, potentially increasing tissue tolerance to persistent lactic acidosis [11];It was found that acetoacetate could improve the memory of Alzheimer's mice by promoting brain-derived neurotrophic factors and inhibiting inflammation [12]. Reduced acetoacetate concentrations in CSF of TBE patients may lead to neurologic damage in TBE patients. Thus acetoacetate, is expected to provide new insights into the treatment of TBE.

5'-deoxy-5'-(methylthio) adenosine (MTA) (FDR<0.05,$\log_2 FC$=-4.15, AUC=0.974) is an endogenous compound produced through polyamine metabolism. It was found that MTA possessed a wide range of neuroprotective activity against different injuries [10]. Significantly reduced MTA level in the cerebrospinal fluid of patients with TBE may be associated with neurologic injury.

we took these two metabolites (acetoacetate and MTA) as combination biomarkers to differentiate TBE patients from non-TBE individuals using the machine-learning model of the random forest (RF) , and AUC of the combination reached 0.995 (95%CI: 0.979-1) in CSF samples from the training set respectively (Figure 2D).

We then tested the RF model in CSF samples. In the CSF validation set, which contained 5 non-TBE patients and 10 TBE patients. The cutoff for the prediction score was set at 0.5, which meant that individuals with a score >0.5 would be diagnosed as TBE and those with a score <0.5 would be classified as non-TBE subjects(Figure 2E), all patients were correctly classified, which demonstrating the availability of the two biomarker combination for TBE distinguish both in serum and CSF samples.

Exploration of the differential metabolites associated with fatal cases in CSF samples

Among the TBE patients providing CSF samples, 5 patients eventually died within 60 days of hospitalization, and the other 72 patients were survived. In order to explore the metabolites associated with poor prognosis in CSF samples, PCA analysis was initially conducted, which showed that the metabolomic data of CSF from fatal group were not resolved from survival group, indicating the similar metabolism changes between the two group(Figure 3A). Then comparing each metabolite between the two groups, 4 differential metabolites (Fetal vs Severe, $|\log_2 FC|>1$,p<0.05,VIP>1) were identified, including kynurenic acid, 5-hydroxyindole-3-acetic acid , DL-tryptophan and indole-3-acrylic acid. Kynurenic acid(KYNA) and 5-hydroxyindole-3-acetic acid were significantly increased in the fatal group, as well as DL-tryptophan and indole-3-acrylic acid decreased in the fatal group (Figure 3B-E).

It was reported that KYN) is associated with CNS inflammation, 5-HIAA is a metabolite of 5-HT that promotes neutrophil recruitment from the peripheral circulation to sites of inflammation, their higher level may contribute to neuroinflammation and possible neurological damage[14]; Given that DL-tryptophan and indole-3-acrylic acid are involved in anti-inflammatory response, their lower concentrations may indicate more hyperinflammation during TBEV infection[10,15,16]. The increased kynurenic acid, 5-hydroxyindole-3-acetic acid and decreased DL-tryptophan,indole-3-acrylic acid in fetal cases suggested that these four metabolites may be associate with poor prognosis of TBE.

Discussion:

Tick-borne encephalitis (TBE) is a severe infectious disease of the central nervous system, revealing of

metabolomic changes in cerebrospinal fluid samples is of great significance for understanding the pathological damage during TBEV infection. However, there have been no report on the analysis of metabolites in cerebrospinal fluid samples from TBE patients yet.

In this study, we depicted the CSF metabolic profile of TBE patients and explored the candidate metabolic biomarkers in CSF samples, which have shown a new perspective to understand this disease better at a new molecular level.

In CSF samples, we found that the significantly altered metabolism pathways mainly involved in tryptophan metabolism, citrate cycle (TCA cycle), and purine metabolism, tryptophan metabolism also involved in differentiating the survival and fatal groups.

Tryptophan (Trp) metabolism primarily involves the kynurenine, 5-hydroxytryptamine, and indole pathways. A variety of bioactive compounds produced via Trp metabolism can regulate various physiological functions, including inflammation, metabolism, immune responses, and neurological function[17].Indoles, the metabolites of tryptophan, can promote intestinal epithelial barrier function and reduces inflammatory responses [15];5-hydroxytryptamine, which uses 5-hydroxytryptophan as precursor, is not only related to the functioning of the central nervous system, but also to the pathogenesis of gastrointestinal inflammation [18];Kynurenine(KYNA), higher level representing more severe neuroinflammation, has been considered as an useful diagnostic and monitoring biomarker for neuroinflammation[19]. In this study, the differential metabolites matched on the tryptophan metabolism pathway were revealed to be tryptophan, 2-(formylamino) benzoic acid, 5-hydroxytryptophan and indole-3-acetic acid; the metabolites associated with poor prognosis of TBE included kynurenic acid, 5-hydroxyindole-3-acetic acid, DL-tryptophan, indole-3-acrylic acid, all were associated with tryptophan metabolism, demonstrated that tryptophan metabolism have played a key role during TBE pathological process. Among the differential metabolites above mentioned, the levels of kynurenic acid and 5-hydroxyindole-3-acetic acid in fetal group were significantly higher than those in the survival group.

Reduced levels of tryptophan and indole-3-acrylic acid (IA) , both possessing anti-inflammatory activity [16,20], were observed in the CSF of TBE patients, and the reduced expression was more pronounced in fatal cases. Indoleamine 2,3-dioxygenase (IDO) is a major enzyme in the catabolism of tryptophan, which regulates immune responses and promotes cancer progression [16]. IDO degrades tryptophan to KYNA, which may cause a decrease in tryptophan and an increase in KYNA [21-22]. In our study, we found a decrease in tryptophan concentration and an increase in kynurenine concentration in fetal cases, which may be associated with IDO activation and hyperimmunity of the organism in severe TBE patients. In a study by M. Holtze et al, the KYNA concentrations were significantly higher in TBE patients than in controls, and the concentration of KYNA varied widely among TBE patients, the more severe, the more increased of KYNA in CSF [19,23], this is consistent with our findings, demonstrating that KYNA can be used as a potential marker to differentiate the severity of TBE.

5-hydroxyindole-3-acetic acid (5-HIAA) is a derivative of serotonin, its level can be used in the clinical diagnosis of neuroblastoma or carcinoid syndrome, as well as essential hypertension, depression, and migraine [24]. Afarideh M et al. found that elevated 5-HIAA concentrations were associated with hypersensitive C-reactive protein (hs-CRP) and low-grade inflammation in metabolic syndrome [25]. The elevated level of 5-HIAA in TBE patients, especially in the fetal patients, demonstrates that the elevated 5-HIAA in the CSF may be associated with inflammation and nerve damage caused by TBEV infection, and 5-HIAA promises to be a potential marker for assessing severity in TBE.

The metabolites matched on the purine metabolism pathway are adenosine, deoxyadenosine, xanthosine, inosine, 2,6-dihydroxypurine, all of these metabolites were reduced in the CSF of TBE patients. Purines play an integral role in cellular processes such as energy metabolism and cell signaling. The purine nucleotides adenine and guanine are key components of RNA and DNA, the need for large amounts of nucleic acids in highly proliferating cells (e.g., cancer, immune cells, and infectious pathogens) can lead to a reduction in purine content [26]. In this study, we found the decreased purine metabolism, which may be due to changes caused by the consumption of large amounts of nucleotides by the virus and immune cell reproduction.

We also found a disturbed TCA cycle in which the matched L-malate concentrations are reduced and cis-aconitic acid, isocitric acid, and alpha-ketoglutaric acid concentrations are increased. The TCA cycle is the hub for the metabolic linkage of sugars, lipids, and amino acids and has a key role in ATP production as well as in the synthesis of the biomolecules needed for viral replication. The final assembly and budding process of enveloped viruses, for instance, require lipids, and the TCA cycle provides the precursor (citrate) for fatty acid synthesis (FAS) [27]. ATP is an energy source for many types of viruses and is used to promote viral replication. Intermediates of the TCA cycle have been shown to bind directly to dendritic cells, causing the production of pro-inflammatory cytokines. It has been proposed that these metabolites released from damaged cells can act as a danger signal to the immune system, promoting a rapid response to pathogens [28].The TCA cycle in TBE patients is altered, probably as a result of immune reaction and energy consuption.

In a mouse brain metabolomics study of zika virus infection, it was found that a dramatic alteration of nicotinamide adenine dinucleotide (NAD+)-related metabolic pathways, including oxidative phosphorylation, TCA cycle and tryptophan metabolism, and the level of malate in the TCA cycle was also significantly decreased, which demonstrated the significant alteration of TCA cycle and tryptophan metabolism caused by Flavivirus [29].

In previous study about the serum metabolism profile investigation, changes in extracellular matrix, complement binding, hemostasis, lipid metabolism, and amino acid metabolism between TBE patients and healthy controls have been demonstrate,the changed pathways including glycerophospholipid metabolism, caffeine metabolism and steroid hormone biosynthesis etc, and multiple phospholipids including bilirubin, LysoPC (18:1[9Z]), palmitic acid, and CL (8:0/8:0/8:0/8:0) etc were significantly different and identified to be candidate serum biomarkers for distinguishing TBE disease from healthy[8, 9].Among which, only 13 metabolites(α-Aspartylphenylalanine, Bilirubin, 5-Hydroxytryptophan ,etc.) were identified to be differential metabolites in current study, demonstrating the very different metabolism change between CSF and serum.

CONCLUSIONS

In this study,we reported a comprehensive CSF metabolomic profile of TBE patients and explored 20 metabolic biomarkers for identifying TBE disease, including acetoacetate, 5'-deoxy-5'-(methylthio)- adenosine, 3-methyl-2-oxobutanoic acid ,etc, and the involved changed metabolism pathways were tryptophan metabolism,purine metabolism and citrate cycle pathways. These findings can improve the understanding of TBE pathogenesis and facilitates target screening for therapeutic inter-vention. Novel biomarkers differentiate between TBE and non-TBE may complement current diagnostic methods.

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Author Contributions

X. Kang and T. Jiang formulated and supervised the project. R. Liang analyzed the data. Y. Li,Y. Gao, S. Zhang,FL.Tan, collected the samples, J. Li, Y. Feng, Yuehong Chen, F. Wang,assisted with the data analysis. All authors contributed to the manuscript revision.

Competing interest

The authors declare no conflict of interest.

Supplementary Information

The data that support the findings of this study are available in the Supporting Information of this article.

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