

Diagnostic performance of miR-21, miR-124, miR-132, and miR-200b serums in post-stroke cognitive impairment patients

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Abstract

Introduction: The diagnosis of post-stroke cognitive impairment (PSCI) mainly depends on neuro-psychological evaluation. It still lacks a sensitive and objective diagnostic biomarker. MicroRNAs (miRNAs, miRs) are novel and potential disease biomarkers. Our aim was to detect which specific miRNA is a good diagnostic biomarker for PSCI.

Material and methods: There were 77 first-ever stroke patients enrolled. Blood samples were collected at 14 days after stroke. Level of serum miR-21, miR-124, miR-132, and miR-200b were determined by quantitative polymerase chain reaction. Mini-mental state examination (MMSE) scale was used to measure the cognitive function of patients. Fractional anisotropy (FA) score of diffusion tensor imaging was applied to detect the alteration of white matter. In addition, the relationship between miRNA level and cognitive status was further explored by correlation analysis.

Results: Finally, 45 PSCI and 32 post-stroke cognitive normality (PSCN) patients were enrolled. The expression of miR-21, miR-132, and miR-200b in PSCI patients was higher than in PSCN patients. In particular, the miR-21 level was substantially correlated with MMSE scores ($r = 0.752, p < 0.001$) and FA value ($r = 0.636, p < 0.001$). Additionally, the diagnostic performance of miR-21 alone or the combination of miR-21 and FA values performed well.

Conclusions: The miR-21 alone or combination of miR-21 and FA values are valuable diagnostic biomarkers in discriminating PSCI from PSCN.

Key words: stroke, cognition, microRNA, diagnosis, biomarkers.

Introduction

Stroke is a common cerebrovascular disease, with high incidence and disability rate, and also is a leading cause of death worldwide. With aging population and an incidence of stroke in younger age [6,8], stroke patients have a longer life expectancy to live with disability, leading to a reduced quality of life. The burden caused by a stroke is expected to be higher in the future. Post-stroke cognitive impairment (PSCI) is one of the common complications of a stroke. There are 51.9% stroke survivors accompanied by cognitive impairment at 6 months after

stroke [24], which is strongly related to poor prognosis of motor and speech deficits, thus prolonging the cost and duration of hospitalization [32].

Currently, magnetic resonance image (MRI) [1] and cognitive assessment scales [18] usually served as diagnostic indicators of PSCI. They are effective, but also have various limitations. Specifically, it is challenging to detect functional changes of patients without brain structural changes in MRI [12]. Cognitive assessment scales have the following limitations: a systematic review identify that there are 25 different types of cognitive assessment scales in clinical practice [17]. There is no consensus which cognitive

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assessment scale is the best screening tool for PSCI. Preferred assessment would depend on purpose of testing [22]. Moreover, there are different versions even for the same scale [21]. Optimal cut-off value of the same assessment tool at different disease stages may be different [30]. Furthermore, cognitive examination is subjective, and clinical applications have found that different people's assessments are likely to produce different results. They are also easily affected by culture, language, education, and other factors. Together, it is necessary to find a more sensitive and objective diagnostic biomarker.

MicroRNAs (miRNAs, miRs) are 20-23 nucleotides non-coding RNAs that negatively regulate gene expression at post-transcriptional level *via* mRNA degradation or translational inhibition [23]. MiRNA is considered a novel and valuable biomarker of disease diagnosis [19]. A total of four miRNAs (miR-21, miR-124, miR-132, and miR-200b) were selected in this study since these miRNAs have been reported to be closely related to stroke and cognitive impairment in previous literature. MiR-21 [38] and miR-200b [16] play a neuro-protective role in stroke. MiR-124 is the most abundant miRNA in the brain [26]. MiR-132 is one of the most studied miRNAs related to cognitive function, possibly by regulating synaptic plasticity [11]. It is not yet known which miRNA can be a diagnostic biomarker in PSCI patients. Here, we characterized differential expression of serum miRNAs between PSCI and post-stroke cognitive normality (PSCN) patients, especially the expressed level of miRNAs. In addition, we detected the link between miRNA expression levels and neuro-imaging diffusion tensor imaging (DTI) results and calculated their diagnostic performance for PSCI patients.

Material and methods

Participants and serum samples

This study was approved by the Ethics Committee of Shanghai University of Medicine and Health Sciences Affiliated Zhoupu Hospital in China (Project ID: 2019-WJW-BX). Written informed consents for participation in this study were obtained from all individuals. Patients with a first-ever stroke were admitted to Shanghai University of Medicine and Health Sciences Affiliated Zhoupu Hospital from January 2019 to December 2019. Finally, a total of 77 patients, including 45 PSCI patients and 32 PSCN patients, completed all mini-mental state examination (MMSE) assess-

ments. Inclusion criteria of PSCI group consisted of: 1) diagnosed with a first-ever stroke based on MRI and clinical evaluation; 2) MMSE scores ≤ 24 ; 3) age older than 18 years old. Exclusion criteria consisted of: 1) a history of stroke; 2) additional comorbidities of nervous system, pregnancy, infectious disease, and cancer. PSCN group had the same inclusion and exclusion criteria, except for MMSE scores (> 24).

At 14th day after stroke onset, peripheral blood (4 ml) was collected from all participants after fasting for 12 hours. Blood samples were centrifuged at 800 g for 10 min at 4°C. About 1 ml of supernatant was then transferred to a clean 1.5 ml centrifuge tube, and was centrifuged again at 1,600 g for 10 min at 4°C to obtain serum. Samples were stored at -80°C until further analysis.

Cognitive function assessment

Cognitive function was evaluated with mini-mental state examination (MMSE) scores [3] at 14th day after stroke. MMSE scores ranged from 0 to 30, consisting of orientation, attention, and calculation, recall, language, and praxis. A higher score indicated better cognitive function. A score greater than 24 showed normal cognitive function, while a score less than or equal to 24 indicated impaired cognitive function. Higher score suggested better cognitive function. All assessments were performed by the same rehabilitation therapist.

Diffusion tensor imaging analyses

To evaluate the alteration of white matter fiber bundles, diffusion tensor imaging (DTI) data were acquired by a 1.5T MRI system (TOSHIBA EXCELART Vantage) equipped with an 8-element receive head and neck coils array. The procedure was performed by a skilled radiologist. DTI images were obtained by applying a single excitation spin-echo planer sequence using the following parameters: repetition time = 12,000 ms, echo time = 100 ms, average number of signals = 40, diffuse b-value = 1,000 s/mm², field of view = 22, matrix size = 128 × 128, and direction number of diffusion sensitive gradient = 6. Fractional anisotropy (FA) value of region of interest (ROI) selected from the infarct brain areas was calculated by a software.

Quantification of miRNAs *via* qRT-PCR

According to the manufacturer's instructions, total RNA was isolated from serum sample using mir-

Table I. Sequences of PCR primers

Primer	Sequence (5'→3')
miR-39	UCACCGGGUGUAAAUCAGCUUG
miR-21	CAACACCAGUCGAUGGGCUGU
miR-124	UAAGGCACGCGGUGAAUGCCAA
miR-132	UACAGUCUACAGCCAUGGUCCG
miR-200b	UAAUACUGCCUGGUAUGAUGA

Table II. Baseline participants' characteristics (mean ± SD)

Variables	PSCN	PSCI
Number of patients	32	45
Sex (M/F)	18/14	23/22
Age (years) ^a	66.28 ±13.97	65.80 ±11.39
Weight (kg) ^a	66.09 ±10.12	57.98 ±9.73***
Height (cm) ^a	169.25 ±5.31	166.51 ±6.39
Systolic blood pressure ^a	142.25 ±18.46	143.76 ±20.51
Diastolic blood pressure ^b	73.59 ±9.04	74.76 ±9.73
mRS ^a	3.19 ±0.97	3.53 ±1.10

^aMann-Whitney U test, ^b Student's t test, *** $p < 0.001$

Vana Paris kit (Ambion, Austin, Texas) and quantified by Nano Drop ultrafine spectrophotometer. Four miRNAs (miR-124, miR-132, miR-200b, and miR-21) were selected for our study by searching and reading the relevant literature. RNA reverse transcription reaction was performed using Bio-Rad reverse transcription kit to synthesize miRNA-specific cDNA. The expression of specific miRNA was detected by quantitative real-time PCR (qRT-PCR) using SYBR GREEN fluorescence system. Briefly, to normalize between samples, miR-39 was added. Cycling conditions for qRT-PCR included pre-denaturation at 95°C for 3 minutes, followed by a total of 40 cycles according to a cycle of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Then, a melt-curve analysis was obtained to evaluate PCR specificity. Relative expression of specific miRNAs was calculated by using $2^{-\Delta\Delta Ct}$ method. All reactions were repeated three times. PCR primers are presented in Table I.

Statistical analysis

All statistical analyses were performed using SPSS 18.0 statistical software, and graphs were generated by GraphPad Prism 8 software. Data were described as mean ± standard deviation (SD). Descriptive statistics, Shapiro-Wilk normality test were used to investigate characteristics of the baseline data. Normal distribution of the data was evaluated by an indepen-

dent-samples' *t*-test, and non-normal distribution data was evaluated by a non-parametric Mann-Whitney test. Similarly, a non-parametric Mann-Whitney test was performed to analyze differentially expressed serum miR-124, miR-132, miR-200b, and miR-21 between PSCI and PSCN patients. To estimate the correlation between miRNAs levels and cognitive status, Spearman's correlation analysis was applied. Furthermore, using a receiver operating characteristic curve (ROC) analysis, the area under the curve (AUC) was calculated to evaluate diagnostic performance of miRNAs. *P*-value < 0.05 was considered statistically significant.

Results

Subject characteristics

A total of 77 stroke patients were involved in this study, including 45 PSCI patients and 32 PSCN patients. There was no significant differences in age, height, modified ranking scale (mRS) scores, and blood pressure among the groups, while PSCI patients had a smaller bodyweight than PSCN patients. Information of these stroke patients are shown in Table II.

Differential expression of miRNAs between PSCI and PSCN patients

To investigate whether these four miRNAs can serve as diagnostic biomarkers of PSCI, qRT-PCR was used to explore the differentially expressed miRNAs. The relative expression of miRNAs is demonstrated in Figure 1. The level of miR-21 showed a higher expression (4.57 ± 2.67 vs. 1.29 ± 1.55 , Mann-Whitney test, $p < 0.001$) in PSCN patients than in PSCI patients. The level of miR-132 (7.39 ± 8.24 vs. 2.88 ± 4.24) and miR-200b (4.98 ± 4.88 vs. 2.41 ± 4.00) was also higher in PSCN patients, respectively (Mann-Whitney test, $p < 0.01$). However, there was no significant difference in the level of miR-124 among PSCI and PSCN patients.

Correlation between miRNAs level and cognitive status

The present study investigated the correlation between the miRNAs level and MMSE scores *via* Spearman's correlation analysis. Results found that a significant positive correlation ($r = 0.752$, $p < 0.001$) existed between the level of miR-21 and MMSE scores (Fig. 2A). The poor positive correlation was observed in miR-132 ($r = 0.319$, Fig. 2B) and miR-200b ($r = 0.379$, Fig. 2C).

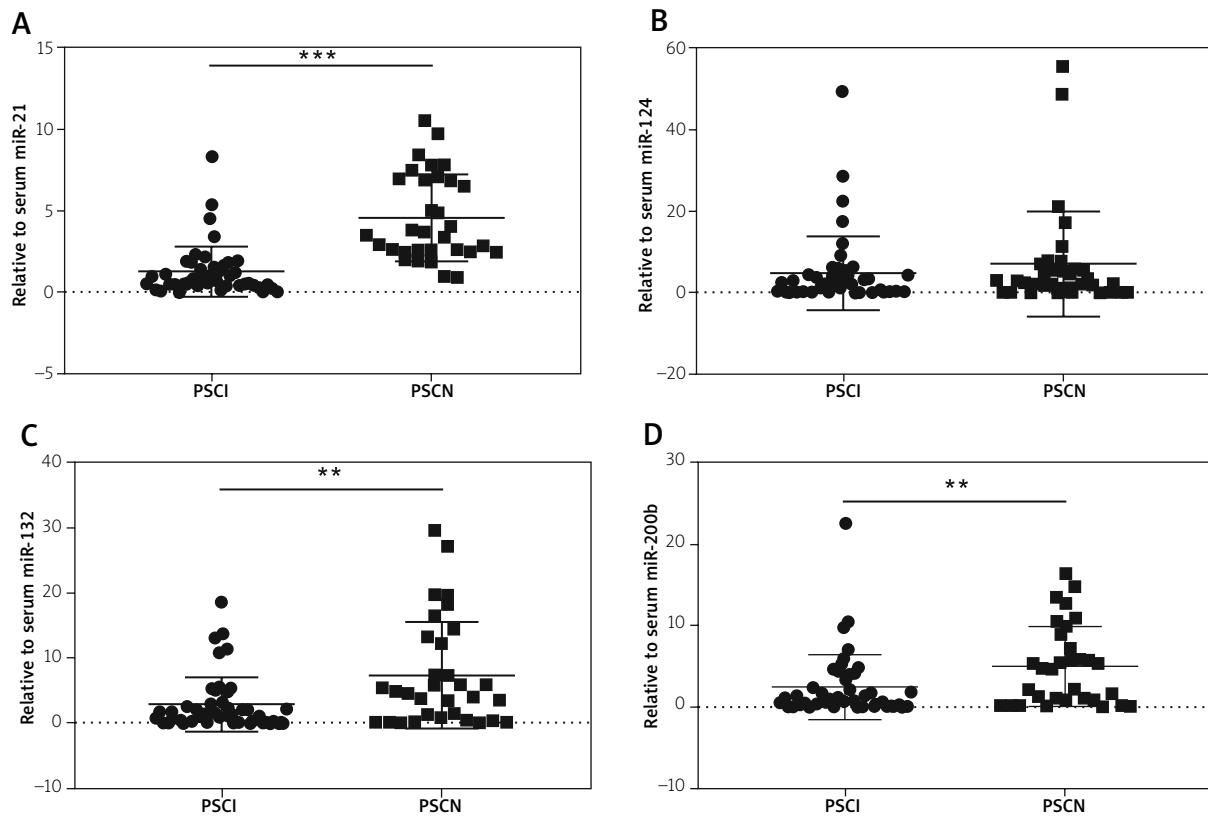


Fig. 1. The differential expression of miRNAs in PSCI patients ($n = 45$) and PSCN patients ($n = 32$). The level of serum miR-21 (A), miR-132 (C), and miR-200b (D) in PSCI patients is higher than in PSCN patients. However, there is no significant difference in the level of miR-124 (B) between PSCI patients and PSCN patients. Data are expressed as mean \pm SD. Mann-Whitney test, *** $p < 0.001$, ** $p < 0.01$.

Correlation between miRNAs level and FA value

To investigate the relationship of miRNA level and brain health in PSCI patients, we conducted a Spearman’s correlation analysis to find the relationship between the miRNAs level and FA value of DTI analysis, which reflected the alteration of white matter fiber bundles. The results found that only miR-21 was closely related with FA value ($r = 0.636$, Fig. 3B), miR-21 was a valuable biomarker to reflect the alteration of white matter.

Diagnostic performance of differentially expressed miRNAs

ROC analysis was performed to detect the diagnostic performance of differentially expressed miRNAs in discriminating PSCI from PSCN. miR-21 performed best and showed the most significant AUC of 0.912. Moreover, miR-132 and miR-200b showed a significant performance with AUC of 0.673 and

0.692, respectively (Fig. 4A). We further explored the diagnostic performance of the combined miRNAs. The results found that miR-200b and miR-132 failed to enhance the diagnostic value of miR-21 (Fig. 4B). The combination of the three miRNAs had the most excellent value, but it was not significant compared with miR-21 alone. Overall, the diagnosis of miR-21 alone was sensitive and efficient in PSCI. The FA value can reflect the change of white matter in the brain, and was also a good diagnostic indicator for the diagnosis of PSCI. Statistical analysis showed that the combined diagnosis of miR-21 and FA value was better (Fig. 4C). These data are shown in Table III. Taken together, results suggested that miR-21 alone, or the combination of miR-21 and FA value could be diagnostic biomarkers for distinguishing PSCI and PSCN.

Discussion

Post-stroke cognitive impairment contributes to patient’s prolonged hospitalization and reduced qual-

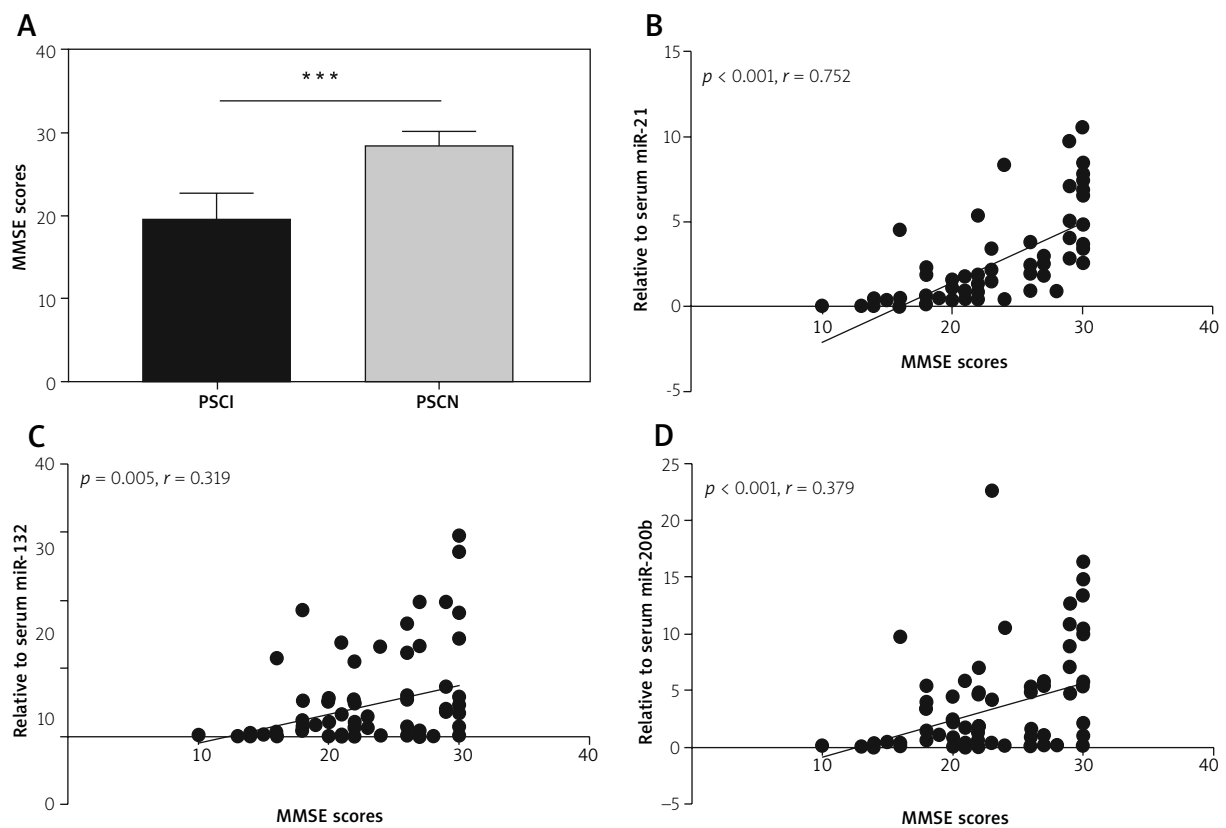


Fig. 2. Correlations between the level of miRNAs and MMSE scores. (A) MMSE scores in PSCI are smaller than those in PSCN. A positive correlation exists between the level of (B) miR-21, (C) miR-132, (D) miR-200b, and MMSE scores. Data are expressed as mean ± SD. Spearman’s correlation analysis ($n = 77$). MMSE – mini-mental state examination.

ity of life [32]. Early diagnosis is necessary. Currently, the clinical diagnosis of PSCI mainly depends on neuro-psychological evaluation and imaging assessment. However, neuro-psychological evaluation is subjective and not yet standardized [4]. Imaging assessment is expensive, and it is difficult to distinguish early cognitive impairment [12]. An early and sensitive diagnosis of PSCI is challenging.

Circulating miRNA are highly stable in the blood. Its’ detection is simple, convenient, and efficient. Recent studies have confirmed that circulating miRNAs are potential biomarkers for diagnosing diseases [27]. miR-29a and miR-146a are potential diagnostic biomarkers in distinguishing Alzheimer’s disease (AD) and vascular dementia (VaD) from healthy control subjects [7,28]. However, less research has focused on detecting potential miRNAs that discriminate PSCI from PSCN. Sessa and colleagues [29] found that higher levels of miR-200b and miR-21 indicate age-related cognitive impairment. Besides,

the higher levels of miR200b, miR21, and miR124 suggest brain stroke. In this study, we confirmed that miR-21, miR-132, and miR-200b were valuable diagnostic biomarkers of PSCI. Furthermore, the level of miR-21 was substantially correlated with MMSE scores, with the best diagnostic performance. As far as we know, this is the first time that miR-21 and miR-200b were reported to serve as a diagnostic biomarkers in PSCI. Our study provides data for similar research in the future.

This study found that miR-21 could be PSCI diagnostic biomarker and correlated with cognitive status. MiR-21 has also been repeatedly proved to be closely related to cognitive function before. The expression of miR-21 was consistently positively correlated with the volume of cerebral hematoma in less than 7 days of cerebral hemorrhage, suggesting that miR-21 can reflect the development process of stroke pathology [25]. Two clinical studies have explored the performance of miR-21 in diagnosing

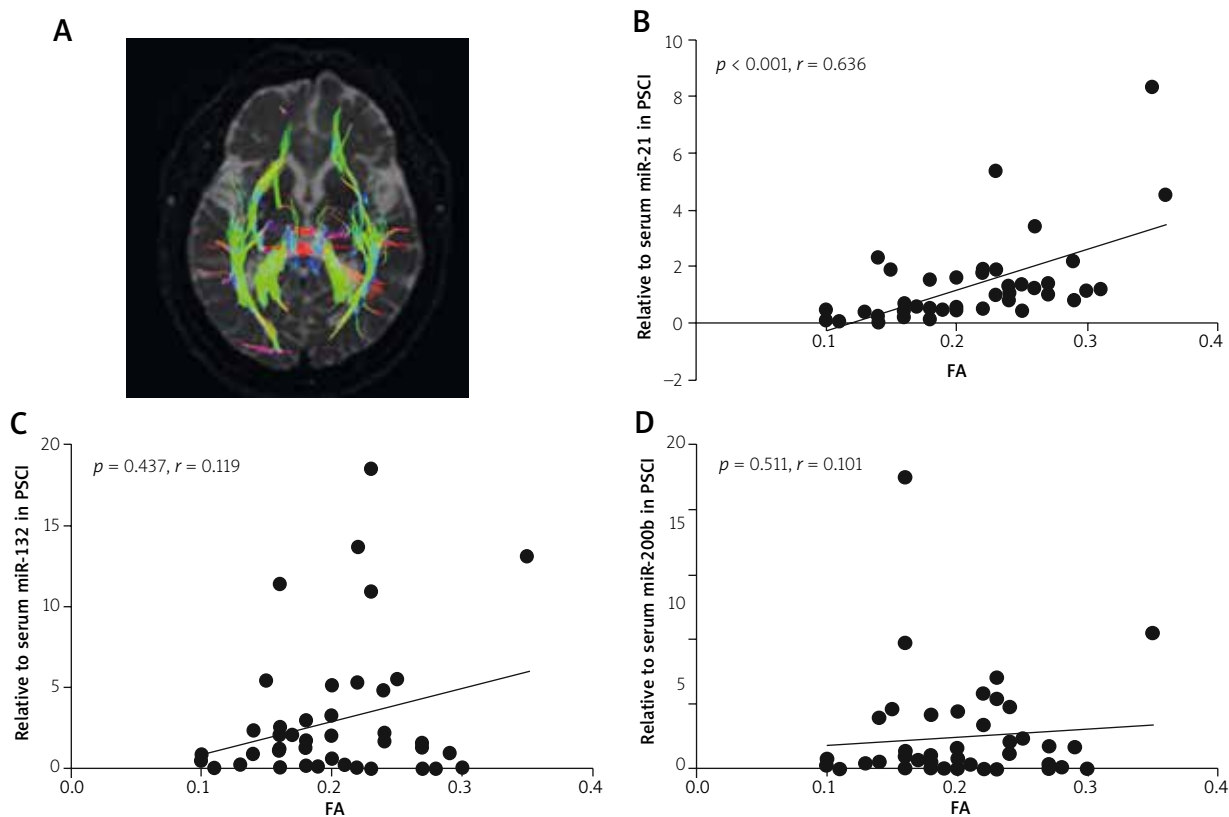


Fig. 3. Correlations between the level of miRNAs and FA value in PSCI patients. **A)** The DTI image shows that the number of nerve fiber bundles in the ipsilateral side (left) of stroke injury is significantly smaller compared with the contralateral side. A positive correlation exists between the level of **(B)** miR-21 and FA value. Data are expressed as mean \pm SD. Spearman's correlation analysis ($n = 45$)

vascular cognitive impairment. Marchegiani *et al.* [20] analyzed the expression differences of miR-21 in the cerebrospinal fluid between vascular cognitive impairment, AD, and cognitive functioning normal groups in a study among 17 VaD patients, showing no significant differences between the three groups. Sorensen *et al.* [31] obtained similar results; they compared the miR-21 expression in AD group and other dementia groups, including vascular cognitive impairment. No significant difference was found in cerebrospinal fluid or blood samples. It is worth noting that this clinical study enrolled only 4 vascular cognitive impairment patients. The results may not be sufficient to prove that miR-21 failed in distinguishing AD and VaD. Overall, current studies have shown limited use of miR-21 as a biomarker for VCI diagnosis. The diagnostic of miR-21 in PSCI has not been studied before. Moreover, miR-21 has been repeatedly proved to be a powerful anti-apoptotic

factor, and plays a neuro-protective role in cerebral ischemic and refusion injury [2,37,38]. Zhou and colleagues [38] found that miR-21 upregulates the level of anti-apoptotic Bcl₂ protein. Similar results were observed by Yang and colleagues [37], who found that miR-21 inhibited apoptosis by increasing the ratio of Bcl₂/Bax via PTEN/Akt-dependent mechanism after cerebral ischemic and refusion injury. Overexpression of miR-21 also significantly alleviated lipid accumulation and inflammatory responses through TLR4-NFkB pathway [9]. Accordingly, miR-21 is considered to be an attractive therapeutic prospect for the treatment of stroke.

Although miR-124 failed to be PSCI diagnostic biomarker, miR-124 was confirmed to be an anti-apoptotic factor. Wang *et al.* [34] found that the overexpression of miR-124 upregulated Bcl₂ protein in PC12 cells to alleviate cell death after ischemic stroke, possibly by activating the PI3K/Akt signaling pathway [34].

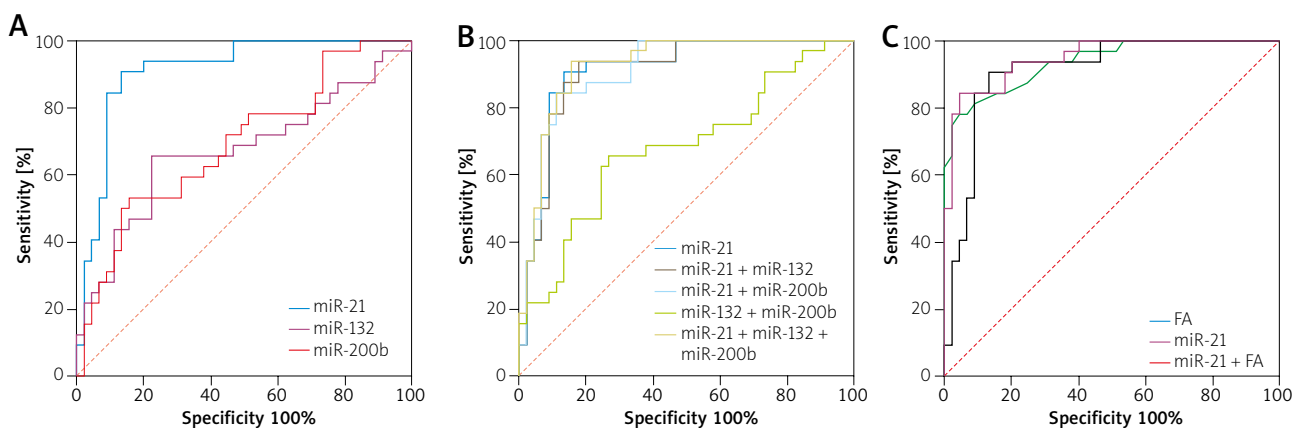


Fig. 4. The diagnostic performance of miRNAs in post-stroke cognitive impairment. **A)** Diagnostic performance of serum miR-21, miR-132, and miR-200b is differently expressed in PSCI patients and PSCN patients. **B)** Diagnostic performance of the combination of miRNAs. **C)** Diagnostic performance of the FA value alone, and the combination of miR-21 and FA value. ROC analysis is applied to evaluate their diagnostic performance in distinguishing PSCI from PSCN.

Table III. Diagnostic performance of miRNAs

miRNA	AUC	95% CI	Cut-off value	Sensitivity (%)	Specificity (%)
miR-21	0.912	0.845-0.979	1.945	90.6	86.7
miR-132	0.673	0.544-0.802	3.348	65.6	77.8
miR-200b	0.692	0.572-0.813	4.666	53.1	84.4
miR-21 + miR-132	0.908	0.840-0.976	0.271	93.8	82.2
miR-21 + miR-200b	0.912	0.848-0.975	0.315	84.4	88.9
miR-132 + miR-200b	0.681	0.556-0.805	0.368	65.6	73.3
miR-21 + miR-132 + miR200b	0.926	0.867-0.984	0.291	93.8	84.4
FA	0.938	0.886-0.989	0.295	78.1	95.6
miR-21 + FA	0.950	0.906-0.994	0.439	84.4	95.6

Additionally, miR-124 plays an important neuro-protective role by repressed NF-κB signaling activation and reactive oxygen species production [35].

MiR-132 and miR-200b are proved to be possible diagnostic biomarkers of PSCI in this study. MiR-132 is a well-known regulator of cognitive capacity, and is necessary for memory formation and retention [10,11]. MiR-132 has been proven to be a risk marker for PSCI [14]. Similar to our study results, the most recent research found that, compared with PSCN patients, the level of miR-132 in cerebrospinal fluid was down-regulated in PSCI patients [36]. However, miR-132 also has been found to up-regulate the serum of PSCI patients relative to PSCN patients [14]. Given that the level of miRNA depends on the time and space, and that the sample size of our study was small, results still need further investigation to confirm our findings. The possible mechanism of miR-132 protecting memory impairment was identified by down-regulating the expression

of Nav1.1 and Nav1.2 [13]. Furthermore, miR-132 has shown to have protective effect on ischemia-induced hippocampal CA1 neuronal death and blood-brain barrier disruption in ischemic stroke [15,39]. Therefore, miR-132 was considered a novel therapeutic target for amelioration of cognitive deficits. MiR-200b was associated with age-related cognitive impairment and stroke consequences in humans [29], and was found to play neuro-protective roles by downregulating prolyl hydroxylase 2 levels in mice [16].

Cerebral white matter fiber bundles play an important role in the transmission of information in each brain region. Vascular lesions may harm neural network structure and function by damaging white matter. Studies have also reported that high white matter signals are predictors of PSCI [5]. These suggest that brain white matter fiber bundles are closely related to cognitive impairment. DTI, one of the best markers associated with cognitive decline, is a mag-

netic resonance technique that can identify the integrity of brain white matter fiber bundles, and FA value is the main parameter that reflects the integrity of white fiber bundles. The higher score of FA value indicates the better integrity of white fiber bundles [33]. This is the first time the relationship between FA value and miRNA expression was investigated.

The present study has the following limitations. Firstly, the current clinical diagnosis of PSCI is mainly done through cognitive assessment scales. Although the cognitive assessment scales are insufficient, MMSE was used as the main diagnostic scale. Therefore, the miRNAs detected in this study can only be used as assisting diagnostic markers. Secondly, MMSE is a robust test, and other more sensitive neuro-psychological tests are lacking to further detect cognitive impairment. Thirdly, the sample size of this study was relatively small, and no power calculation was included. Also, hemolysis is an important questionable subject in a miRNA study, but we did not record the hemolysis data in detail. Finally, performing array-based technique helps screen for probable and possible miRNAs. However, we mainly obtained candidates miRNAs according to the literature.

In summary, miR-21, miR-132, and miR-200b are valuable diagnostic biomarkers of PSCI. Especially the miR-21 alone, or the combination of miR-21 and FA value could be diagnostic biomarkers for distinguishing PSCI and PSCN.

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Disclosure

The authors report no conflict of interest.

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