Original paper

Folia Neuropathologica

The diagnostic value of serum miR-17-92 cluster in ischemic stroke

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Folia Neuropathol 2024; 62: 1-9 *DOI: https://doi.org/10.5114/fn.2024.138680*

Abstract

Introduction: *Ischemic stroke (IS) is a prevalent disease that poses a significant threat to human life and is responsible for a substantial financial burden. Research has established the crucial role of the miR-17-92 cluster in lung cancer, cardiovascular diseases, and traumatic brain injury. Despite this, few research studies had fully detected the potential of the miR-17-92 cluster as a novel circulating marker for diagnosing IS.*

Material and methods: *miR-17-92 cluster expression in IS was investigated using GSE117064 dataset via bioinformatics analysis. Moreover, qRT-PCR was conducted to further verify miR-17-92 cluster expression in 58 IS individuals and 50 healthy controls (HCs). These cluster members were examined regarding their potential for detecting and diagnosing IS using the ROC method.*

Results: *The expression level of serum miR-20a-5p, miR-19a-3p, miR-18a-5p, and miR-19b-3p was considerably lowered in IS in contrast with HC in both the GSE117064 cohort and clinical cohort. Moreover, these four miRNAs had a fair performance in IS detection. Thereafter, a diagnostic model based on these aforementioned four miRNAs was developed by logistic regression, which had an AUC of 0.974 in the ROC curve. This diagnostic module was verified using the GSE117064 dataset. Further analysis demonstrated an increasing level of the aforementioned miRNAs in day-7 IS patients compared with day-1 IS patients.*

Conclusions: *This research verified the downregulation of the miR-17-92 cluster in IS. This diagnostic model enrolling four cluster members may be a promising biomarker for IS detection.*

Key words: *miR-17-92 cluster, serum, diagnostic biomarker, ischemic stroke.*

Introduction

Stroke, one of the most common diseases, could cause substantial financial burden [20]. In 2019, 12.2 million cases were estimated to be diagnosed with stroke, making it the second most prevalent cause of mortality worldwide [18]. Ischemic stroke (IS) comprises 85% of all stroke cases [14]. The long-term prognosis and life quality of IS are not satisfactory and one of the reasons is the lack of satisfactory diagnosis and treatment methods [19,27]. Ischemic stroke lacks a cost-effective method for its diagnosis and prognosis, except for magnetic resonance imaging and computed tomography scans [3]. An effective strategy for diagnosing IS would not only facilitate early detection but would also guide its treatment, ultimately improving the prognosis of patients. Therefore, the search for novel, non-invasive, relatively economical diagnostic methods for IS is essential. Some circulating biomarkers, interleukin-6, matrix metallopeptidase 9, and C-reactive protein (CRP), have been reported as diagnostic biomarkers in IS [9].

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Received: 10.10.2022, **Accepted:** 07.08.2023, **Online publication:** 2024

However, the specificity and sensitivity of the diagnostic biomarker from body fluids remain questionable.

Interaction between miRNAs and mRNAs may result in mRNA degradation or translation inhibition [1,2]. Interestingly, due to the stability in circulating blood, serum miRNAs have gained interest as a novel marker to detect many diseases, including cancer, IS, and sepsis. miRNA clusters contain many miRNAs transcribed from a single primary transcript. Previous studies have indicated a similarity in function for the miRNAs within the cluster. The miR-17-92 cluster, which includes six miRNAs (miR-19b-3p, miR-20a-5p, miR-19a-3p, miR-18a-5p, miR-17-5p, miR-92a-3p), play a vital function in multiple disorders, including lung cancer, cardiovascular diseases, and traumatic brain injury [10]. Moreover, the miR-17-92 holds promise as an indicator for gastric adenocarcinoma and lung cancer diagnosis [29]. Prior research has implied the predictive role of miR-19a-3p in cerebral ischemia [13]. However, few studies have fully explored the possibility of miR-17-92 as circulating markers for diagnosing IS.

miR-17-92 cluster expression in IS was investigated using GSE117064 dataset *via* bioinformatics analysis. After evaluating this serum miRNA cluster in IS concerning its diagnosis potential, a diagnostic model containing four miRNAs was developed. In order to further confirm the resulting outcomes, another verification set was utilized. These results may provide more evidence about the diagnostic methods for IS.

Material and methods

Enrolment of participants and data acquisition

A total of 58 IS patients and 50 healthy controls (HCs) from the Maoming Petrochemical Hospital from July 2018 to June 2020 were enrolled in this research. The serum IS samples were acquired before the patients received any treatment and 7 days after the onset of the disease. Table I showed the clinical characteristics of IS and HC. In terms of age or gender distribution, no considerable variation was noted between IS and HC groups ($p > 0.05$). The provision of the consent form was necessary for inclusion in the study. The research was subjected to approval by the Ethics Committee of the Maoming Petrochemical Hospital and was performed based on the Declaration of Helsinki. As per the committee regulations, the process of specimen collection was carried out. miRNA-sequencing data were obtained from the GSE117064 dataset, containing 173 IS cases and 1612 HC cases. Data preprocessing was executed through the limma package, which was also utilized for the statistical analyses. The normalization of the data was executed through the robust multi-array average (RMA) method. A limma analysis was used to identify genes with a false discovery [16,22]. Further analysis of the standardized and log2-transformed miRNA expression data was executed.

Collection and processing of serum sample

Peripheral blood (15 ml) from IS and HC was collected with subsequent centrifugation within 2 h at 4°C, first at 1000 g for 10 min and then for a further 5 min at 15,000 g. The extracted serum was then purified. Adding synthetic *Caenorhabditis elegans* miR-39 (cel-miR-39, obtained from RiboBio, China) in a concentration of 2 µl of 10 nmol/l to the samples as a normalization control facilitates the qRT-PCR procedures.

RNA extraction and qRT-PCR

Based on the specified protocol, TRIzol LS isolation kit (Thermo Fisher Scientific, USA) was utilized for the extraction of the total RNA of the serum sample. Afterwards, 30 µl RNase-free water was utilized for resuspension of the total RNA, along with subsequent storage of the samples at –80°C for further study.

Table I. Participant characteristics

Characteristics	Healthy control (*n* = 50)	Ischemic stroke patients (*n* = 58)	*P*-value
Gender (female), *n* (%)	19 (38)	22 (37.9)	1.000
Age (years), mean ±SD	65.86 ±14.44	66.29 ±13.41	0.872
BMI (kg/m^2), mean ±SD	25.25 ±4.65	25.62 ±3.84	0.644
Hypertension, *n* (%)	9 (18)	16 (27.6)	0.262
History of heart failure, *n* (%)	5 (10)	8 (13.8)	0.768
History of atrial fibrillation, *n* (%)	4 (8)	16 (27.6)	0.012
History of hyperlipidaemia, *n* (%)	6 (12)	20 (34.5)	0.007
History of type 2 diabetes mellitus, *n* (%)	6 (12)	11 (19.0)	0.429
Current smoking, *n* (%)	7 (14)	13 (22.4)	0.324

The extracted samples were evaluated concerning their concentration and purity through the NanoDrop 2000 spectrophotometer (NanoDrop, USA). To amplify the miRNA, the Bulge-Loop miRNA qRT-PCR Primer Set (RiboBio, China) was employed to obtain the specific reverse transcription primer. Subsequently, qRT-PCR was conducted in 384-well plates on LightCycler 480 Real-Time PCR System (Roche Diagnostics, Germany) utilizing the SYBR Green qPCR kit (SYBR Pre-mix Ex Taq II, TaKaRa). The procedure consisted of an initial step at 95°C for 30 s (denaturation), with subsequent 35 cycles at 95°C, 60°C, and 70°C for the respective periods of 10 s, 20 s, and 10 s. Ultimately, melting curve analysis was employed to assess the specificity of the PCR product. All miRNA expressions were normalized to exogenous control cel-miR-39 before further analysis. Each reaction was repeated thrice or more.

Statistical analysis

The distribution of samples was examined by means of the Shapiro-Wilk normality test. Percentages were utilized to present the categorical variables and clinical data, which were examined through the Chi-square test. However, the Student's *t*-test was utilized to assess the variation between IS and HC in the expression of serum miRNA. Additionally, stepwise logistic regression analysis was employed for developing the diagnostic model. Moreover, the paired-*t* test was performed for comparative assessment of day-1 and day-7 stroke. The diagnostic value of miRNA expressions in predicting IS was evaluated using receiver operating characteristic (ROC) curves. The data were analyzed through GraphPad Prism software v 8.0 (GraphPad Software, USA) and SPSS v 22.0 (IBM Corporation, USA).

Results

Assessment of the expression and diagnostic ability of the members of the miR-17-92 cluster in ischemic stroke in the GEO dataset

miRNA-isoform sequencing data were isolated from GSE117064 dataset (Fig. 1A). Low fold change (FC) value implies a considerable variance between the IS and HC groups regarding the miRNA expression, allowing for better differentiation between the groups. The resulting data exhibited that the level of miR-19a-3p ($p = 0.0343$), and miR-17-5p, miR-18a-5p, miR-20a-5p, miR-19b-3p and miR-92a-3p (all $p < 0.001$) was suppressed in IS vs. HC (Fig. 1A). The area under the curve (AUC) values of the ROC curve to distinguish IS from HC were 0.539, 0.698, 0.619, 0.671, 0.654, and 0.736, respectively (Fig. 1B). These data imply that the miRNAs exhibit moderate discrimination ability in distinguishing IS from HC.

Assessment of the expression and diagnostic capacity of miR-17-92 cluster members in ischemic stroke in the clinical dataset

Table I showed the characteristics and demographics of the participants. In terms of gender, age, and body mass index (BMI), no considerable variation was depicted between the IS group and the HC group. Notably, atrial fibrillation ($p = 0.012$) and hyperlipidaemia ($p = 0.007$) were determined to function as risk factors for IS. The level of the members of the miR-17-92 cluster was examined in the serum of individuals with IS (day 1) and HC. The resulting data implied that in contrast with the HC group, the level of miR-92a-3p ($p = 0.0423$), as well as of miR-18a-5p, miR-20a-5p, miR-19a-3p, and miR-19b-3p (all $p < 0.001$), were considerably lowered in the IS group (Fig. 2A). However, no remarkable difference was obtained in miR-17-5p between the IS group and the HC group (Fig. 2A, $p = 0.4428$). Afterwards, the capability of the miR-17-92 cluster to distinguish individuals with IS from HC was examined through ROC curves. The data indicated that miR-19b-3p (AUC = 0.904) had the highest accuracy in diagnosing IS among these six members comprising the miR-17-92 cluster, followed by miR-20a-5p, miR-19a-3p, and miR-18a-5p, with an AUC of 0.861, 0.831, and 0.815, respectively (Fig. 2B). However, the capacity of serum miR-17-5p (AUC = 0.551) and miR-92a-3p (AUC = 0.661) to diagnose and detect IS is relatively low (Fig. 2B).

Construction and validation of the diagnostic model for ischemic stroke

Usually, achieving high sensitivity and specificity, simultaneously, is not possible for a single biomarker. Thus, in order to obtain maximum diagnostic ability, a diagnostic model was developed by combining several miRNAs with stepwise logistic regression analysis. Hence, four miRNAs (miR-20a-5p, miR-19b-3p, miR-19a-3p, and miR-18a-5p) that depicted a fair performance in detecting IS were selected for the diagnostic model construction (AUC > 0.8). As a result, all these four miRNAs were enrolled in this diagnostic model with the formula: Logit(P) = (–4.113) * ExpmiR-18a-5p + (–6.633) * ExpmiR-19a-3p + (–9.141) * ExpmiR-19b-3p + (–7.320) * ExpmiR-20a-5p. The data demonstrated that the AUC of this diagnostic model containing four miRNAs was 0.974 ($p < 0.0001$, sensitivity = 98.2%, specificity = 94.0%) (Fig. 3A). Additionally, this diagnostic model was verified further using the GSE117064

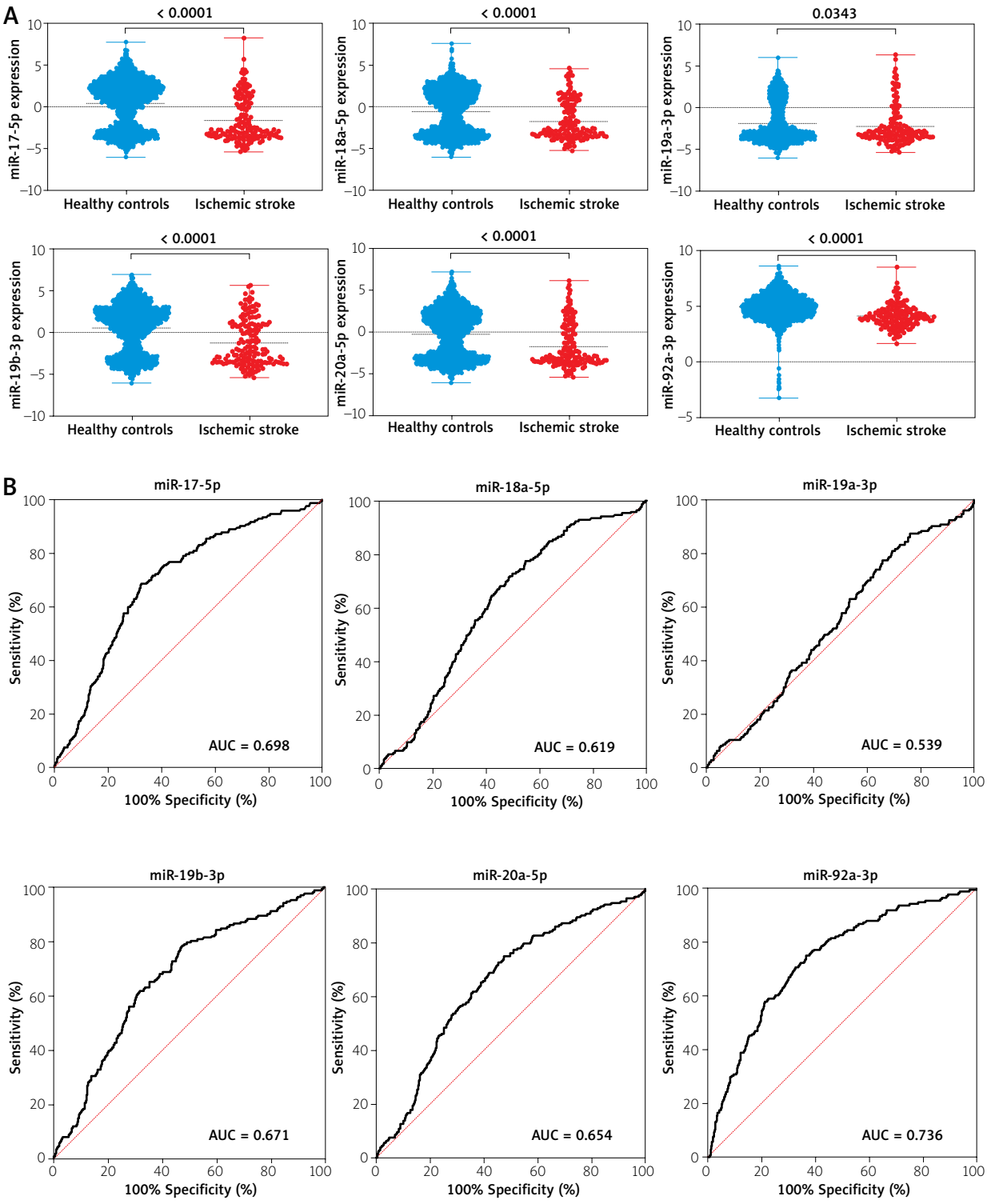

Fig. 1. Expression profiles and diagnostic significance of the members of the miR-17-92 cluster in ischemic stroke (IS) in GSE117064 cohort. **A**) The level of members of the miR-17-92 cluster in ischemic stroke patients and healthy controls. **B**) The ROC curve of the members of the miR-17-92 cluster in the diagnosis of IS.

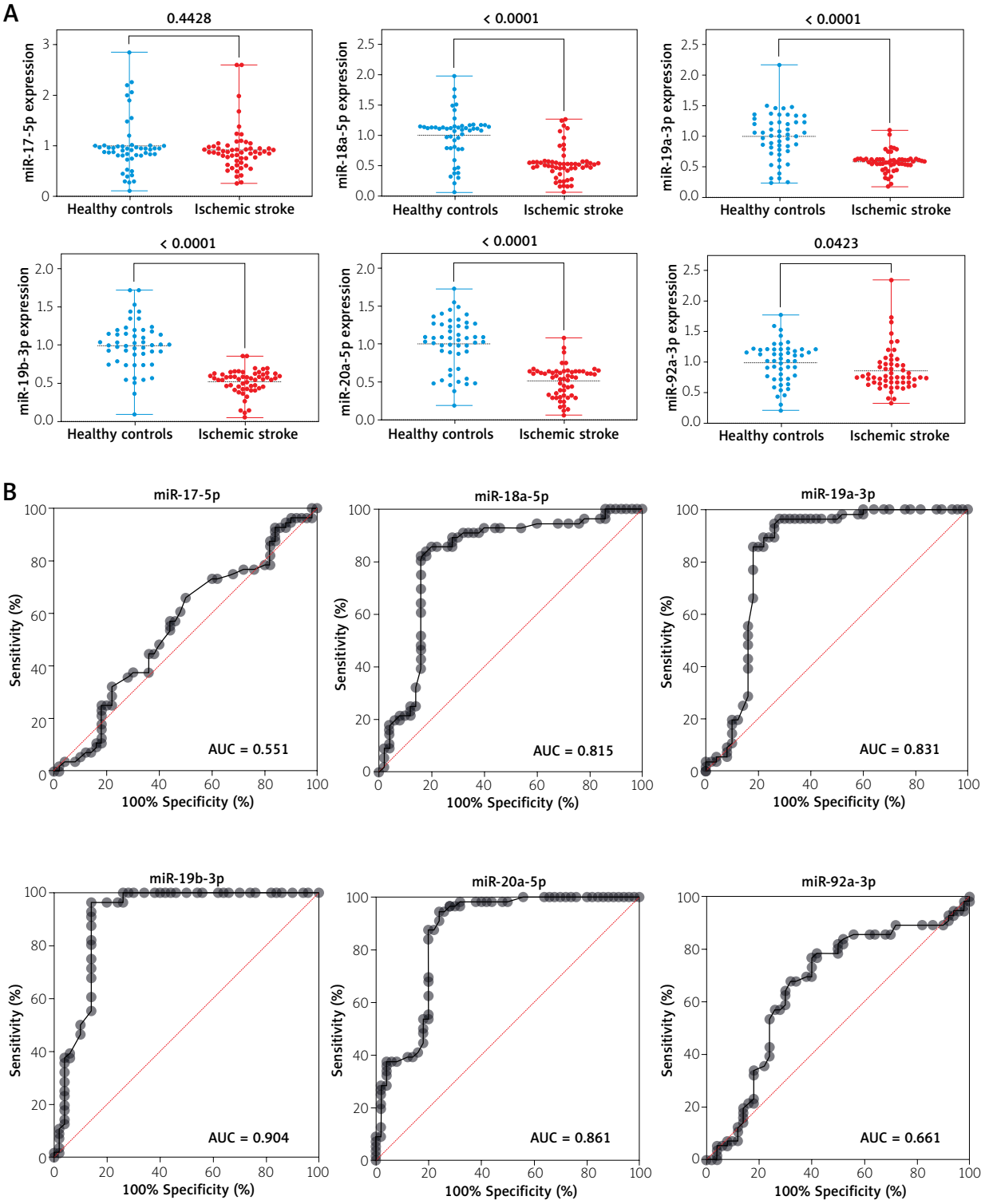

Fig. 2. Expression profiles and diagnostic significance of members of the serum miR-17-92 cluster in ischemic stroke (IS) in the clinical cohort. **A**) The level of the miR-17-92 cluster in ischemic stroke patients and healthy controls. **B**) The ROC curve of the miR-17-92 cluster in the diagnosing IS.

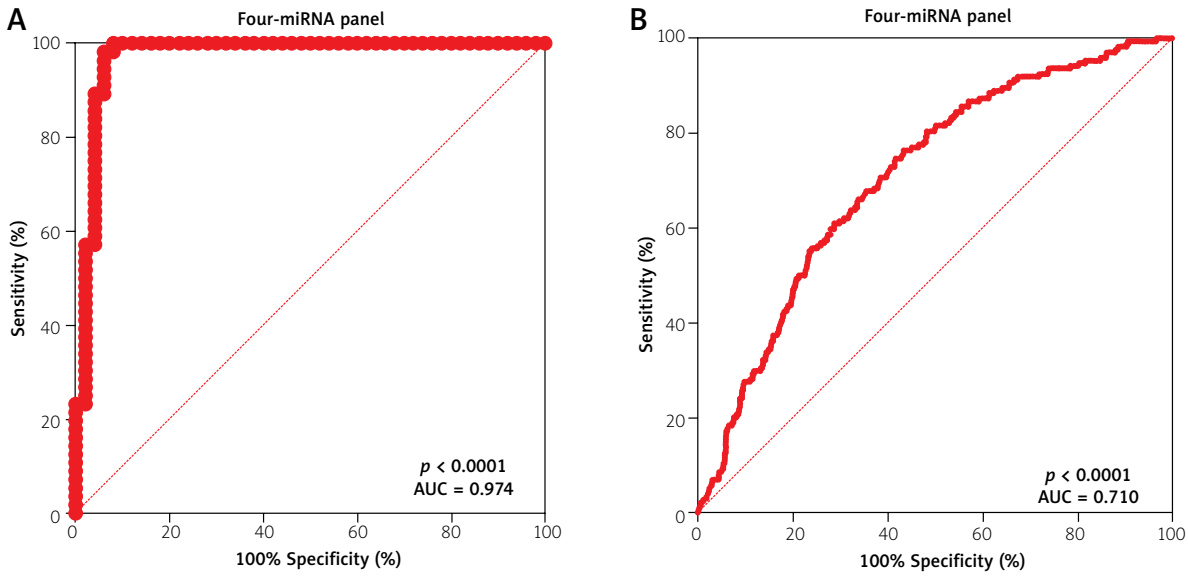

Fig. 3. Development and validation of the diagnostic model for ischemic stroke (IS). **A**) The ROC curve of the diagnostic model in the clinical cohort. **B**) The ROC curve of the diagnostic model in the GSE117064 cohort.

dataset. The resulting data implied that this four-miRNAs diagnostic model had a fair performance in detecting IS with an AUC value of 0.710 (Fig. 3B).

Evaluation of the expression of miR-17-92 cluster members in day-7 ischemic stroke in the clinical dataset

The serum of day-7 IS patients was also obtained. Interestingly, the data suggested an increasing level of miR-19b-3p (Fig. 4B), miR-20a-5p (Fig. 4C, both $p < 0.001$), miR-19a-3p (Fig. 4D, $p = 0.0014$), and miR-18a-5p (Fig. 4E, $p = 0.0036$) expressed in day-7 IS patients compared with day-1 IS patients. However, no remarkable variation was noted in the expression of miR-17-5p (Fig. 4A, $p = 0.5599$) and miR-92a-3p (Fig. 4F, $p = 0.2520$) between day-7 and day-1 IS patients. These data further confirmed the promising potential of these four miRNAs in IS detection and diagnosis.

Discussion

The significance of the diagnosis of liquid biopsy had been highlighted in the last decade. Liquid biopsy could act as a promising method for precise treatment and monitoring of treatment response in real-time [23]. Moreover, liquid biomarkers in serum could be used for the diagnosis of many diseases [10,24]. Though some liquid biomarkers, including ceramides, sphingosine-1-phosphate, and FABP4 [4,12,30], have been identified for the diagnosis of IS, they have not yet been used in clinical diagnosis.

The polycistronic miRNA cluster, miR-17-92, is a highly conserved region. It is located within the open reading frame 25 on the 13th chromosome (C13orf25) [21]. Increasingly, research has revealed dysregulation in the level of this cluster in many diseases, including cancer, anxiety, depression, and cardiac ischemic disease [11,17,31]. Moreover, miR-17-92 diagnostic potential has been extensively explored in retinoblastoma, lung cancer [5,15]. The assumption that this cluster could function as novel circulating markers for the diagnosis of IS remains to be comprehended. Hence, this research dealt with clarifying the diagnostic value of the miR-17-92 cluster in IS.

The miR-17-92 cluster was examined in the serum, which indicated that the level of miR-20a-5p, miR-19a-3p, miR-18a-5p, and miR-19b-3p was remarkably lowered in IS in contrast with HC in both GSE117064 and clinical cohorts. Further diagnostic analysis revealed that the miR-19b-3p (AUC = 0.904) had the highest accuracy in diagnosing IS among the six members of the miR-17-92 cluster, followed by miR-20a-5p, miR-19a-3p, and miR-18a-5p, with respective AUCs of 0.861, 0.831, and 0.815. Therefore, these four miRNAs depicted a fair performance in IS detection. Prior studies have proposed these miRNAs in the role of diagnostic biomarkers for certain types of disorders. Previous research has indicated that circulating miR-19b-3p functions as potential diagnostic biomarkers for acute myocardial infarction [25]. Another study revealed the promise that circulating miR-19b-3p holds as a novel indicator for the diagnosis of diabetic cardiomyopathy [8]. A pilot study suggested plasma miR-20a-5p as

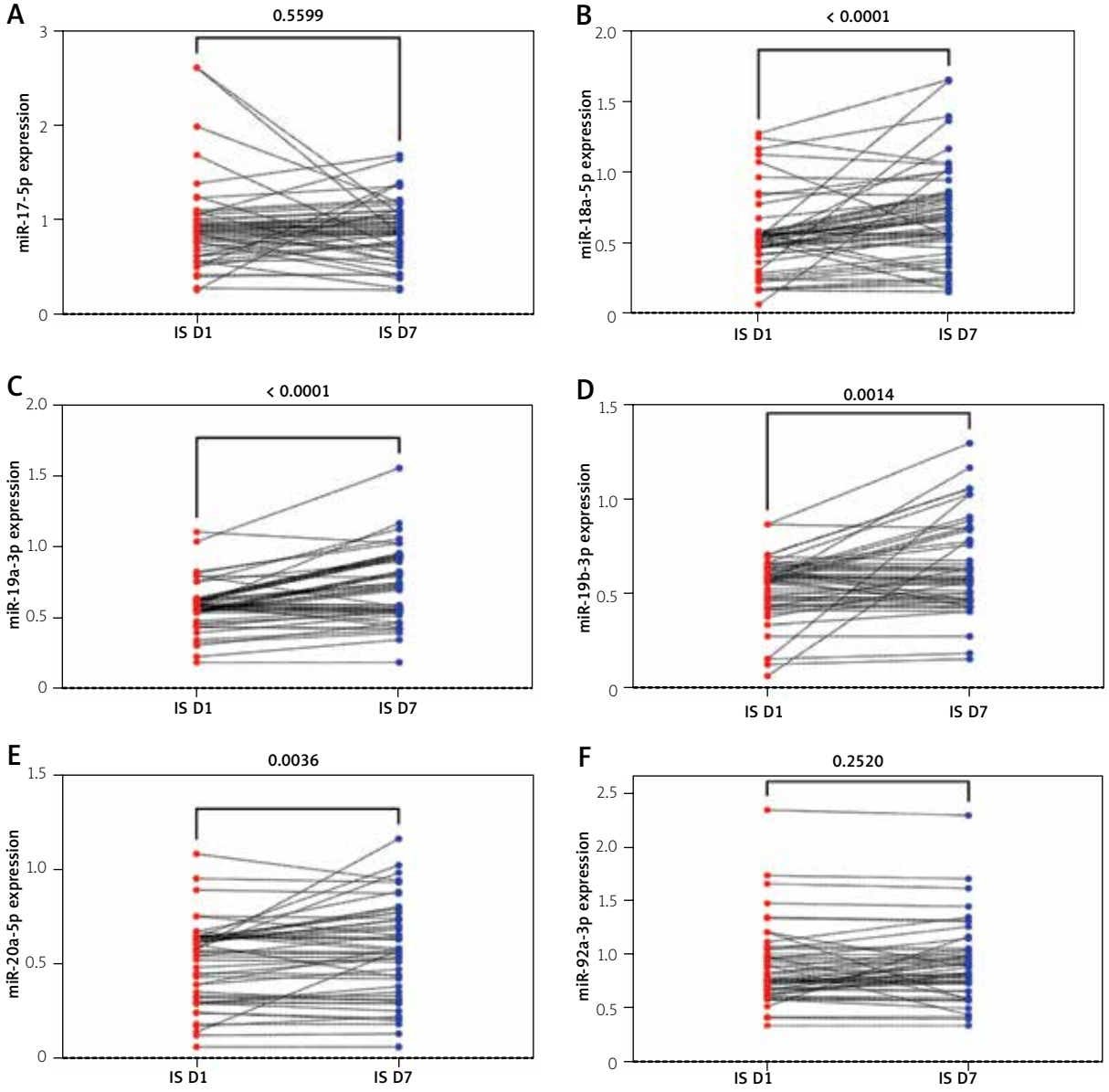

Fig. 4. The expression levels of members of the miR-17-92 cluster in day-1 and day-7 ischemic stroke (IS) in the clinical dataset. The relative level of miR-17-5p (**A**), miR-19b-3p (**B**), miR-20a-5p (**C**), miR-19a-3p (**D**), miR-18a-5p (**E**) and miR-92a-3p (**F**) in day-1 and day-7 IS patients.

a promising non-invasive indicator for diagnosing male infertility [7]. Moreover, circulating miR-19a-3p could distinguish gastric cancer patients from healthy controls [6].

There is no doubt that many serum miRNAs could serve as markers for IS diagnosis. For instance, RNA-Seq analysis data of prior research revealed the potential of circulating miR-125b-5p as indicators for IS diagnosis. Another study suggested miRNA-155 being a plasma marker for IS [28]. Moreover, circulatory miRNA-4271 could differentiate individuals with IS from healthy controls with high accuracy [26]. However, few diagnostic models combining various miRNAs for IS have been constructed to date. In this study, a diagnostic model was developed based on the four selected miRNAs for IS by logistic regression, with an AUC of 0.974 in the ROC curve. However, when the model was applied to a publicly available dataset (GSE117064), the AUC value of the ROC curve was only 0.710. This discrepancy in performance may be attributed to two main

factors. Firstly, the GEO data were generated using high-throughput sequencing, which enables the simultaneous detection of numerous gene expression but may also introduce technical variability that can reduce the accuracy of the results. Secondly, only 108 cases (58 IS patients and 50 healthy controls) were used for validation, and this small sample size results in reducing the reliability and generalizability of the data. Thus, these data suggested that the four-miRNA diagnostic model had a fair performance in IS detection. However, further studies should be performed to verify our findings using a larger sample size.

Conclusions

Our research verified the downregulation of the serum miR-17-92 cluster in IS. The developed diagnostic model incorporating four cluster members has the potential to function as an indicator for the detection of this disease.

Funding

This work was supported by the Natural Science Foundation of Zhejiang Province (LY19H090005), Science and Technology Project of Jinhua City (2018-3-025, 2021-3-151), and Basic Public Welfare Research Project of Zhejiang Province (LGF20H090006).

Disclosure

The authors report no conflict of interest.

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