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Increased plasma levels of miR-124-3p, miR-125b-5p and miR-192-5p are associated with outcomes in acute ischaemic stroke patients receiving thrombolysis

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HIGHLIGHTS

- Nineteen known microRNAs associated with outcome were discovered via RNA sequencing.
- Three microRNAs were validated using qRT-PCR and showed higher predictive efficiency.
- Two microRNAs were associated with stroke severity.

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ABSTRACT

Background and aims: Circulating microRNAs (miRNAs) have recently emerged as promising biomarkers for acute ischaemic stroke (AIS). However, the expression profiles of miRNAs in AIS patients receiving intravenous thrombolysis, and their associations with outcome have not been investigated.
Methods: In a prospective cohort study, a total of 84 AIS patients, who received intravenous thrombolysis (21.4% received combined reperfusion therapy) and completed 3 month follow-up visits, were included. Favourable and unfavourable outcomes were defined as modified Rankin Scale (mRS) scores of 0–1 and 2–6, respectively. Plasma samples were collected at 24 h after thrombolysis. We used RNA sequencing to study miRNA profiles in 5 patients with unfavourable outcomes and 5 matched patients with favourable outcomes. Differentially expressed miRNAs were further validated in all cohorts using quantitative real-time polymerase chain reaction assays.
Results: After identification and validation, we found that miR-124-3p, miR-125b-5p and miR-192-5p levels were higher in patients with unfavourable outcomes than in patients with favourable outcomes. Logistic regressions and receiver-operating characteristic curve analyses demonstrated that these altered miRNAs may function as predictive biomarkers for outcome in AIS patients receiving thrombolysis, whether combined with endovascular thrombectomy or not. In addition, miR-124-3p and miR-125b-5p were closely associated with stroke severity.
Conclusions: A set of circulating microRNAs (miR-124-3p, miR-125b-5p and miR-192-5p) are associated with unfavourable 3 month outcomes and might have clinical utility in AIS patients receiving thrombolysis.

1. Introduction

Acute ischaemic stroke (AIS) is a common cause of disability and mortality worldwide that has become a serious social burden [1,2]. Timely recombinant tissue plasminogen activator (rt-PA) therapy within 4.5 h after the onset of AIS has been proven as an effective therapy that improves the prognosis and reduces disability [3,4]. Early prediction and risk stratification in AIS patients receiving thrombolysis are important for stroke treatment and management [4].

MicroRNAs (miRNAs) are small non-coding RNAs approximately 22

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nucleotides in length [5]. Molecular biology discoveries have revealed that miRNAs are distinctly determined in the central nervous system and participate in the pathogenesis and progression of neurological diseases [5,6]. In addition, miRNAs are stably detectable in the circulation even under unfavourable physiological conditions, allowing their potential use as circulating biomarkers [6,7]. Recent studies have reported specific circulating miRNA markers related to the diagnosis, severity, and prognosis of AIS [8–10].

A number of studies have demonstrated that treatment with rt-PA after AIS extensively alters the miRNA expression profile, both in the brain tissue of animals [7,11,12] and in the peripheral blood of patients [13]; these findings indicate the possibility that specific miRNAs could be used as prognostic biomarkers in AIS patients receiving thrombolysis. In addition, the identification of blood-based miRNAs might contribute to a better understanding of the mechanisms of rt-PA treatment and their potential use as innovative targets in the treatment of AIS.

Therefore, in the present study, we sought to determine whether miRNAs are early prognostic biomarkers in plasma samples from AIS patients receiving thrombolysis. We used miRNA sequencing technology and then validated the differentially expressed miRNAs using quantitative real-time PCR (qRT-PCR). In addition, the validated miRNAs were measured in some samples from patients before intravenous rt-PA treatment. Moreover, clinical relevance and bioinformatics analyses of the validated miRNAs were also performed.

2. Patients and methods

2.1. Study population

The study included consecutively enrolled AIS patients receiving rt-PA within 4.5 h after their symptom onset at the Department of Neurology of Shanghai Ninth People's Hospital between February 2015 and April 2018. Experienced neurologists evaluated the patients based on their history, physical examination, and imaging examination and determined their National Institute of Health Stroke Scale (NIHSS) score before thrombolysis after entering into emergency room. We enrolled and excluded patients according to the 2013 guidelines for the early management of patients with AIS [14]. In addition, we excluded patients with severe renal, liver or thyroid failure, acute infectious diseases, rheumatic immune or haematologic diseases, cancer, or significant missing data.

The study was approved by the local ethics committee and was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki as well as institutional guidelines. All patients or their immediate family members were informed about the study and provided written informed consent.

2.2. Clinical assessment and data collection

Demographics, clinical features, and cardiovascular risk factors were extracted from the patients' medical records. Hypertension, diabetes mellitus, dyslipidaemia, coronary heart disease, and atrial fibrillation were indicated based on the hospital examination, previous history and use of medications, as previously described [15,16]. A control computed tomography (CT) was performed 24 h after rt-PA infusion or condition aggravation. Stroke aetiology was classified according to the TOAST classification criteria [17]. Infarct volumes were quantified via magnetic resonance imaging (diffusion-weighted or fluid-attenuated inversion recovery), and the modality with the largest infarct size was used for volumetry. An experienced neuroradiologist blinded to the clinical presentations and laboratory examinations calculated the infarct volume based on the sum of every slice infarction area multiplied by the slice thickness [9].

A total of 94 AIS patients who received thrombolysis were prospectively included. At 3 months after stroke onset, outcomes were assessed through telephone follow-up by a trained investigator who was unaware of the patients' parameters. Eighty-four patients completed the follow-up, and 10 patients were lost to follow-up because their phone numbers were wrong or the calls were unanswered. The baseline characteristics of the 84 patients did not significantly differ from those of the 94 patients (Supplementary Table S1). Functional outcomes were determined using the modified Rankin Scale (mRS). Favourable and unfavourable outcomes were defined as mRS scores of 0–1 and 2–6, respectively [3,18]. Poor outcomes indicating that some help was required with activities of daily living were assigned an mRS score > 2 [19].

2.3. Sample collection and RNA isolation

Blood samples from all patients at 24 h after thrombolytic treatment were collected in tubes with EDTA anticoagulant. In addition, 32 of the 84 AIS patients had blood samples before thrombolysis in emergency room. All visibly haemolysed samples were excluded. Plasma separation was achieved by centrifugation at 1600*g* for 10 min at 4 °C. The plasma was aliquoted and frozen at −80 °C until RNA extraction. Total RNA, including miRNA, was extracted from the plasma using an miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany; catalogue number 217184).

2.4. RNA sequencing and data analysis

Small RNA sample prep kits used to prepare miRNA libraries were provided by OE Biotech (Shanghai, China). 3′ and 5′ RNA adaptors were ligated using T4 RNA ligase. Subsequently, reverse transcription and amplification were conducted. Next, 22 nt ~30 nt RNA fragments were isolated via RNA gel electrophoresis. Subsequently, Hiseq X Ten (Illumina, San Diego, CA) was used for cluster generation. Then, after removing the adaptor sequences and filtering the low-quality reads, clean reads were processed for computational analysis. Various 'mappings' were performed using unique seqs against pre-miRNA and mature miRNA sequences listed in the latest release of the miRbase v.21 database (http://www.mirbase.org/) [20]. Unannotated small RNAs were analysed using the miRDeep2 algorithm to predict novel miRNAs [21]. miRNA expression was calculated by transcript per million (TPM), TPM is equal to the number of reads per miRNA matched/all reads × 1000000. The R package EdgeR was used to identify differential expression of the miRNAs [22]. These procedures were performed by OE Biotech.

2.5. qRT-PCR validation

Differentially expressed miRNAs were validated using qRT-PCR. Reverse transcription reactions were performed using an miScript reverse transcription kit (Qiagen; 218161). A SYBR Green PCR kit (Qiagen; 218073) and specific miRNA primers (TIANGEN, Beijing, China) were used to confirm specific miRNAs. For there is no clear consensus on what should be used as an internal reference for miRNA expression profiling in body fluids (such as plasma). Synthetic *C. elegans* miR-39 (cel-miR-39, Qiagen; 219610) was added to the plasma samples as an exogenous control in the extraction process. The threshold cycle (CT) values of cel-miR-39 are shown in Supplementary Table S2. All samples were repeated in triplicate. The CT values were determined automatically by the instrument. The data were normalized to cel-miR-39 according to the formula $2\exp[-(CT_{microRNA} - CT_{cel\text{-}miR\text{-}39})]$ and were compared as previously described [23]. The experimental processes were performed according to the manufacturer's instructions.

2.6. Target gene prediction and bioinformatics analyses

The target genes of differentially expressed miRNAs that were verified in this study were predicted by Miranda [24] with the following parameters: $S \geq 150$, $\Delta G \leq -30$ kcal/mol and demand strict 5′ seed pairing. S refers to single-residue-pair match scores in the matching region, and ΔG refers to the free energy of double chain formation.

Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of miRNA target genes were

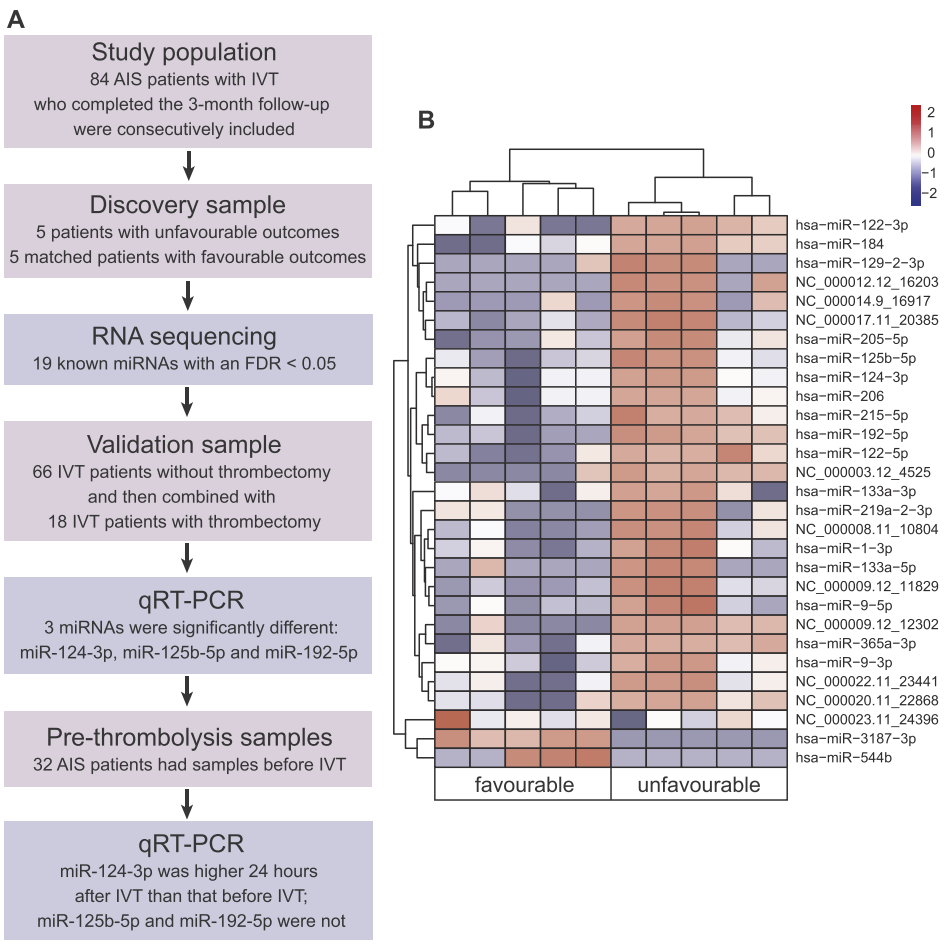


Fig. 1. Study profile and clustering heat map of differentially expressed miRNAs from RNA sequencing.
(A) Study workflow. (B) Nineteen known miRNAs and 10 newly predicted miRNAs showed an FDR < 0.05 (normalization algorithm EdgeR) according to different outcomes. The fold change corresponding to the colour intensity indicates the mean expression. The red colour denotes an increase, and the blue colour denotes a decrease. AIS, acute ischaemic stroke; IVT, intravenous thrombolysis; FDR, false discovery rate; qRT-PCR, quantitative real-time polymerase chain reaction.

performed based on the hypergeometric distribution using R. GO and KEGG analysis results were obtained and are shown in Supplementary Fig. S1 and Fig. S2.

2.7. Statistical analyses

The normality of the data was evaluated by the Shapiro-Wilk test. Continuous variables are described as the means ± standard deviation (SD) or the medians and interquartile range (IQR); these data were compared using Student's *t*-test, Mann-Whitney *U* test, or paired *t*-test, as appropriate. The distribution of the miRNA data approximated a normal distribution after $\log_{10}$ transformation. Categorical variables are given as frequencies (percentages) and compared using the Pearson chi-square test or Fisher's exact test. The predictive values of the miRNAs were assessed by a receiver-operating characteristic (ROC) curve analysis with area under the curve (AUC). The cut-off value of each biomarker was determined using the Youden index. Accommodating covariates methodology was used to determine whether the addition of other covariates significantly influence the discriminatory accuracy [25]. Binary logistic regression analysis was used to combine different parameters to obtain a generalized linear model. The prediction probabilities were calculated according to regression equations and the corresponding probability was used as an additional parameter. The additional parameter was further assessed by ROC analysis. Correlation analysis was performed using the nonparametric Spearman's rank correlation test. A multiple logistic regression analysis for variables with p values < 0.05 according to a univariate analysis was performed. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA), Stata version 14.0 (College Station, TX, USA), and MedCalc 12.5 (MedCalc Software, Ostend, Belgium). A p value < 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the patients

Because we wanted to focus mainly on the relationship between miRNAs and prognosis in AIS patients receiving thrombolysis, we enrolled 84 patients with follow-up data. The characteristics of the 84 patients are shown in Supplementary Table S1. The mean age was 68.3 ± 12.6 years, and 70.2% were male. The median NIHSS upon admission was 5 (2, 12), and the infarct volume was 9.6 (2.3, 31.7) cm^3. Eighteen patients received subsequent endovascular thrombectomy after rt-PA infusion. According to TOAST classification, the stroke aetiologies included large-artery atherosclerosis (34.5%), cardioembolism (21.4%), small-vessel occlusion (31.0%), and other determined or undetermined aetiologies (13.1%).

3.2. Discovering differentially expressed miRNA profiles by sequencing

To determine the profile of circulating miRNAs associated with functional outcomes in AIS patients who received thrombolysis, we first performed RNA sequencing in discovery samples. Studies have determined that endovascular thrombectomy is an effective treatment for AIS [26]. To avoid potentially confounding effects on the results, we did not include patients receiving combined endovascular thrombectomy in the discovery samples.

At the stage of the sequencing analyses, in order to eliminate confounding factors as much as possible, we carefully screened and selected similarly matched patients with similar conditions on admission but with different outcomes. Five patients with unfavourable outcomes and 5 patients with favourable outcomes at 3 months were enrolled

Table 1
Baseline characteristics of the patients for discovery and validation.

	Discovery samples		Patients without thrombectomy		All patients	
	Favourable	Unfavourable	Favourable	Unfavourable	Favourable	Unfavourable
Demographic characteristics						
Total, n	5	5	48	18	55	29
Age, years	72.4 ± 13.8	71.8 ± 11.0	65.2 ± 10.5	71.6 ± 16.1	66.3 ± 10.9[a]	72.2 ± 14.8[a]
Male, n (%)	4 (80)	4 (80)	34 (70.8)	14 (77.8)	38 (69.1)	21 (72.4)
Stroke risk factors, n (%)						
Hypertension	4 (80)	5 (100)	43 (89.6)	16 (88.9)	46 (83.6)	24 (82.8)
Diabetes mellitus	1 (20)	1 (20)	17 (35.4)	8 (44.4)	19 (34.5)	12 (41.4)
Dyslipidaemia	3 (60)	3 (60)	34 (70.8)	11 (61.1)	39 (70.9)	17 (58.6)
History of stroke	0 (0)	1 (20)	4 (8.3)	4 (22.2)	5 (9.1)	7 (24.1)
Coronary heart disease	1 (20)	1 (20)	8 (16.7)	4 (22.2)	12 (21.8)	7 (24.1)
Atrial fibrillation	0 (0)	0 (0)	7 (14.6)	3 (16.7)	11 (20.0)	9 (31.0)
Smoking history	2 (40)	2 (40)	23 (47.9)	6 (33.3)	25 (45.5)	7 (24.1)
Alcohol consumers	1 (20)	2 (40)	20 (41.7)	3 (16.7)	20 (36.4)	5 (17.2)
Medication use, n (%)						
Hypertension medication	3 (60)	4 (80)	32 (66.7)	11 (61.1)	34 (61.8)	16 (55.2)
Diabetes medication	1 (20)	1 (20)	14 (29.2)	3 (16.7)	14 (25.5)	4 (13.8)
Lipid-lowering medication	1 (20)	2 (40)	16 (33.3)	5 (27.8)	19 (34.5)	6 (20.7)
Anticoagulant medication	0 (0)	0 (0)	2 (4.2)	1 (5.6)	2 (3.6)	2 (6.9)
Laboratory parameters						
HbA1c, %	5.6 (5.5, 6.5)	5.8 (5.4, 7.9)	6.0 (5.6, 7.7)	6.3 (5.5, 7.0)	6.0 (5.6, 7.1)	6.3 (5.7, 6.9)
Homocysteine, μmol/L	15.6 (12.0, 16.2)	17.5 (10.5, 18.4)	12.8 (9.8, 15.7)	13.3 (9.5, 19.2)	12.8 (9.7, 15.7)	13.3 (9.5, 18.8)
CRP, mg/L	3.3 (3.2, 3.6)	3.0 (2.2, 4.8)	3.3 (3.0, 5.1)	4.3 (3.1, 12.0)	3.3 (3.0, 6.2)	4.7 (3.2, 9.9)
Stroke evaluation						
NIHSS before thrombolysis	5 (2, 8)	7 (3, 9)	3 (2, 5)[a]	7 (5, 9)[a]	4 (2, 9)[a]	9 (6, 18)[a]
Infarct volume, cm^3	5.0 (0.1, 10.0)	31.3 (17.5, 41.7)	5.8 (0.92, 14.5)[a]	41.0 (9.3, 115.5)[a]	7.1 (1.2, 19.6)[a]	30.7 (8.0, 82.4)[a]
ICH after 24 h, n (%)	0 (0)	1 (20)	5 (9.3)	6 (22.2)	5 (9.3)	6 (22.2)
Time from onset to thrombolysis, min	203 (136, 252)	124 (107, 180)	154 (115, 189)	146 (107, 207)	147 (105, 180)	146 (107, 207)
Endovascular thrombectomy, n (%)	0 (0)	0 (0)	7 (12.7)[a]	11 (37.9)[a]	7 (12.7)[a]	11 (37.9)[a]
TOAST subtype, n (%)						
Large-artery atherosclerosis	2 (40)	2 (40)	16 (33.3)	6 (33.3)	18 (32.7)	11 (37.9)
Cardioembolism	0 (0)	1 (20)	3 (6.2)	4 (22.2)	8 (14.5)	10 (34.5)
Small-vessel occlusion	3 (60)	1 (20)	22 (45.8)	4 (22.2)	22 (40.0)	4 (13.8)
Other determined/undetermined	0 (0)	1 (20)	7 (14.6)	4 (22.2)	7 (12.7)	4 (13.8)
mRS on day 90, points	0 (0, 1)[a]	3 (2, 3)[a]	0 (0, 1)[a]	3 (3, 4)[a]	0 (0, 1)[a]	3 (3, 4)[a]

The values are presented as the mean ± SD or median (interquartile range) for continuous variables and as a number (percentages) for categorical variables.
[a] Reflects $p < 0.05$.
Favourable and unfavourable outcomes were defined as mRS scores of 0–1 and 2–6, respectively.
mRS, modified Rankin Scale; HbA1c, glycated haemoglobin; CRP, C-reactive protein; NIHSS, National Institutes of Health Stroke Scale; ICH, intracranial haemorrhage; rt-PA, recombinant tissue-type plasminogen activator; TOAST: Trial of Org 10172 in Acute Stroke Treatment.

(Fig. 1A). These patients were matched for age, sex and NIHSS score (8 was the cut-off). No significant differences were noted in the characteristics between the groups (Table 1). Illumina sequencing analyses of small RNAs isolated from plasma samples provided median read counts of 19.2 (IQR 18.8, 22.1) million in patients with favourable outcomes and 19.6 (IQR 19.2, 21.7) million in patients with unfavourable outcomes (Supplementary Table S3). A total of 468 (IQR 444, 688) known miRNAs and 228 (IQR 215, 474) predicted miRNAs were found in patients with favourable outcomes, and 554 (IQR 523, 613) known mature miRNAs and 272 (IQR 253, 324) predicted miRNAs were found in patients with unfavourable outcomes (Supplementary Table S4). The miRNA reads per sample (raw and normalized) are presented in Supplementary Table S5. Hierarchical clustering was performed with differentially expressed miRNAs according to different outcomes (Fig. 1B).

Twenty-nine miRNAs showed a false discovery rate (FDR) adjusted $p < 0.05$. Among them, 19 known miRNA (17 miRNAs were upregulated, and 2 miRNAs were downregulated) and 10 predicted miRNAs (9 miRNAs were upregulated and 1 miRNA was downregulated) were detected, in patients with unfavourable outcomes compared with those in patients with favourable outcomes (Supplementary Tables S6 and S7).

3.3. Validation of differentially expressed miRNAs

We validated the differentially expressed miRNAs using qRT-PCR in the all cohort (Fig. 1A). miRNAs were chosen for validation if the mean of the normalized values was greater than 1.0 in both groups. Using this criterion, 10 known miRNAs and 2 predicted miRNAs in the discovery samples were selected for validation (Supplementary Tables S6 and S7). First, we validated these 12 miRNAs using qRT-PCR in patients without endovascular thrombectomy. Of the 10 miRNAs, miR-124-3p and miR-125b-5p were significantly higher in the patients with unfavourable outcomes than in the patients with favourable outcomes ($p = 0.007$ and $p = 0.038$; Table 2, Fig. 2A and B). In addition, there was a trend of increasing miR-192-5p levels in the unfavourable groups ($p = 0.082$; Table 2, Fig. 2C). For the other 9 miRNAs, we did not find significant differences (Supplementary Table S8).

A total of 18 patients who received thrombolysis combined with endovascular thrombectomy were included in the entire research cohort. We detected the above 3 miRNAs in these patients and then pooled the data of all patients with or without thrombectomy as a reperfusion treatment set. Compared with patients with favourable outcomes, patients with unfavourable outcomes were older, had higher NIHSS scores and larger infarct volumes (Table 1). The expression of all 3 above mentioned miRNAs was significantly higher in patients with unfavourable outcomes than that in patients with favourable outcomes (miR-124-3p: $p = 0.001$; miR-125b-5p: $p = 0.004$; and miR-192-5p: $p = 0.038$; Table 2; Fig. 2D, E and F). After adjustment for age and NIHSS score, a multivariate logistic regression analysis indicated that increased miR-124-3p, miR-125b-5p and miR-192-5p levels were closely associated with unfavourable outcomes (miR-124-3p: $p = 0.021$; miR-125b-5p: $p = 0.009$; and miR-192-5p: $p = 0.029$; Table 2).

Table 2
Relation of validated microRNAs to functional outcomes.

	Patients without thrombectomy			All patients				
	Favourable	Unfavourable	*p* value	Favourable	Unfavourable	*p* value	Adjusted OR (95% CI)[a]	Adjusted *p* value[a]
miR-124-3p	−2.99 ± 0.53	−2.57 ± 0.53	0.007	−2.91 ± 0.57	−2.46 ± 0.56	0.001	1.831 (1.096, 3.058)	0.021
miR-125b-5p	−3.00 ± 0.32	−2.78 ± 0.47	0.038	−2.98 ± 0.34	−2.72 ± 0.42	0.004	2.002 (1.188, 3.373)	0.009
miR-192-5p	−2.03 ± 0.32	−1.85 ± 0.46	0.082	−2.01 ± 0.36	−1.82 ± 0.41	0.038	1.727 (1.058, 2.818)	0.029

Log_{10}-transformed values were used.
The data were normalized to cel-miR-39 according to the formula $2exp[-(CT_{microRNA} - CT_{cel\text{-}miR\text{-}39})]$.
[a]Adjusted for age and NIHSS score upon admission.
OR, odds ratio; CI, confidence interval.

3.4. ROC curve analysis of validated miRNAs as prognosis markers

Given the upregulated levels of miR-124-3p, miR-125b-5p and miR-192-5p in patients with unfavourable outcomes, we then determined their role as prognostic markers using a ROC curve. In all patients, we found that miR-124-3p, miR-125b-5p and miR-192-5p had good diagnostic accuracy for the presence of an unfavourable outcome (Fig. 3A). In addition, the 3-miRNA model (miR-124-3p, miR-125b-5p and miR-192-5p) signature showed greater discriminatory ability, with an AUC of 0.803 (95% CI: 0.691–0.888, $p < 0.001$), a sensitivity of 88.00% and specificity of 65.22% (Fig. 3A). Additionally, the ROC curve adjusted for age and/or sex did not change the conclusions (Supplementary Table S9 and Supplementary Fig. S3).

Furthermore, the AUC value for the NIHSS score for predicting an unfavourable outcome increased from 0.762 (95% CI: 0.646–0.855) to 0.838 (95% CI: 0.732–0.915) after combination with these 3 miRNAs ($p = 0.135$).

Similar results were found when we assessed the predictive efficiency of these 3 miRNAs for the presence of a poor outcome (Fig. 3B).

3.5. Determination of validated miRNAs before thrombolysis

For a time-course analysis, we determined the miR-124-3p, miR-125b-5p and miR-192-5p levels before thrombolysis in 32 of the 84 AIS patients (Fig. 1A). miR-124-3p was significantly higher 24 h after thrombolysis than that before thrombolysis ($p < 0.001$, Supplementary Table S10). No differences in miR-125b-5p and miR-192-5p levels were found before thrombolysis and 24 h after thrombolysis (Supplementary Table S10).

3.6. Association of validated miRNAs with stroke severity

We determined whether miR-124-3p, miR-125b-5p and miR-192-5p levels were correlated with stroke severity in all included patients. Stroke severity was assessed by the NIHSS score upon admission and infarct volume. miR-124-3p and miR-125b-5p levels were correlated with NIHSS scores upon admission ($R = 0.382$, $p = 0.001$; and $R = 0.343$, $p = 0.002$) and infarct volume ($R = 0.377$, $p = 0.006$; and $R = 0.302$, $p = 0.033$).

4. Discussion

In this study, we successfully identified and validated the distinctive expression of 3 miRNAs (miR-124-3p, miR-125b-5p and miR-192-5p) that were associated with functional outcomes in AIS patients receiving thrombolysis, whether combined with endovascular thrombectomy or not, based on sequencing technology and individual qRT-PCR validation. Combined with these miRNAs may tend to help improve the discriminatory ability of NIHSS scores in predicting an unfavourable outcome ($p = 0.135$). In addition, miR-124-3p and miR-125b-5p were closely associated with stroke severity.

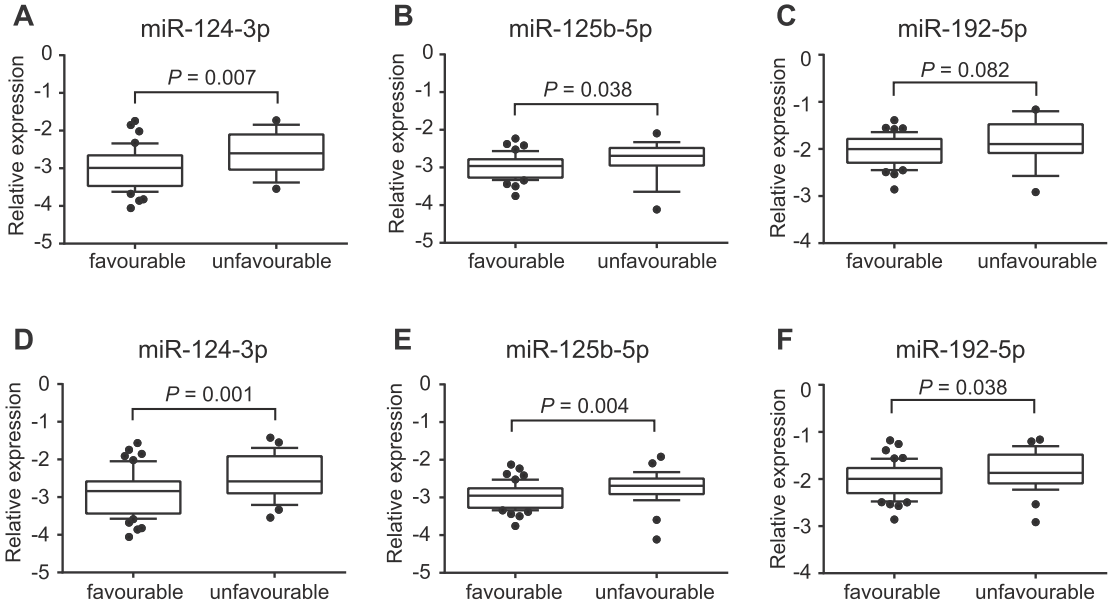
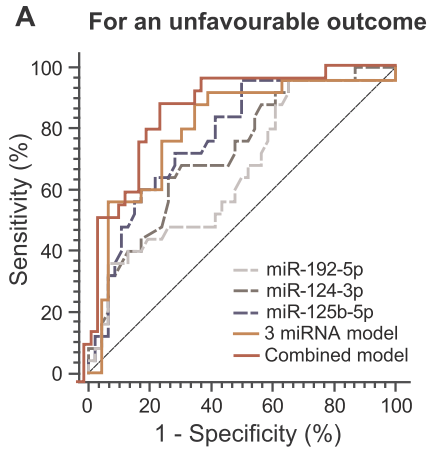
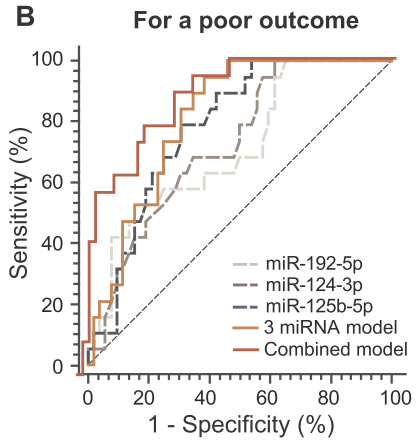


Fig. 2. Validation and replication of miR-124-3p, miR-125b-5p and miR-192-5p.
(A–C) Data for patients receiving intravenous thrombolysis without endovascular thrombectomy (48 *vs.* 18). (D–F) Data for all patients (55 *vs.* 29). The data were normalized to an endogenous control (cel-miR-39) according to the formula $2exp[-(CT_{microRNA} - CT_{cel\text{-}miR\text{-}39})]$. The box plots show the expression levels, and the bars indicate the 10th and 90th percentiles. Differences were compared by Student's *t*-test.



Predictors	SE / SP (%)	ROC curves		
		AUC	95% CI	*P* value
miR-192-5p	92.86 / 35.85	0.647	0.533 - 0.750	0.026
miR-124-3p	66.67 / 69.23	0.707	0.594 - 0.804	< 0.001
miR-125b-5p	92.86 / 46.00	0.729	0.616 - 0.823	< 0.001
3 miRNAs model	88.00 / 65.22	0.803	0.691 - 0.888	< 0.001
Combined model	88.00 / 73.91	0.838	0.732 - 0.915	< 0.001

3-miRNA model included the 3 microRNAs.
Combined model included the 3 microRNAs and NIHSS score.
Abbreviations: SE, sensitivity; SP, specificity; AUC, area under the curve; ROC, receiver-operating characteristic; 95% CI, 95% confidence interval.



Predictors	SE / SP (%)	ROC curves		
		AUC	95% CI	*P* value
miR-192-5p	95.45 / 37.29	0.685	0.572 - 0.784	0.007
miR-124-3p	95.24 / 39.66	0.720	0.607 - 0.815	< 0.001
miR-125b-5p	86.36 / 55.36	0.736	0.624 - 0.829	< 0.001
3 miRNAs model	94.74 / 61.54	0.813	0.703 - 0.896	< 0.001
Combined model	89.47 / 69.23	0.862	0.760 - 0.933	< 0.001

3-miRNA model included the 3 microRNAs.
Combined model included the 3 microRNAs and NIHSS score.
Abbreviations: SE, sensitivity; SP, specificity; AUC, area under the curve; ROC, receiver-operating characteristic; 95% CI, 95% confidence interval.

Fig. 3. Prognostic accuracies of the validated microRNAs as predictors of an unfavourable outcome or a poor outcome at 3 months after stroke.
(A) An unfavourable outcome was defined as a modified Rankin Scale score of 2–6 (55 *vs.* 29). (B) A poor outcome was defined as a modified Rankin Scale score of 3–6 (61 *vs.* 23).

Several studies have highlighted miRNA-mediated regulatory networks in cerebral ischaemia [27], and circulating miRNAs can be utilized as potential biomarkers for AIS detection and evaluation [8–10]. However, systematic characterization studies of the circulating miRNA profiles associated with AIS patient prognosis have rarely been conducted. In addition, recanalization via intravenous thrombolysis or in combination with endovascular thrombectomy has become to be the most effective strategy for AIS worldwide [3,4,28]. However, patients with similar symptoms and severities may have different prognosis after recanalization. Certain miRNA profiles are altered after rt-PA infusion for AIS [12,13,29]; but whether miRNA expression is associated with the severity and outcome of AIS patients receiving thrombolysis has not been previously investigated.

miR-124-3p, a brain-enriched miRNA [30], has been proposed as a marker of ischaemic brain damage. Previous studies have reported that increased levels of miR-124-3p in plasma could predict 3-month mortality and morbidity rates early after the onset of AIS [13], and miR-124-3p levels in cerebrospinal fluid are associated with infarct size in AIS patients [31]. In addition, patients with high levels of miR-124-3p after cardiac arrest were at high risk for poor neurologic outcomes and death [32,33]. No basic experimental study of miR-124-3p in AIS has been reported so far. Increased miR-124-3p levels in microglial exosomes after traumatic brain injury can inhibit neuronal inflammation and contribute to neurite outgrowth [34].

A previous study found that miR-125b-5p has potential clinical utility as a diagnostic marker for acute IS, and when combined with other miRNAs, it showed higher sensitivity than even multimodal CT [9]. The possible role of miR-125b-5p in AIS has not been completely elucidated. The experimental data suggest that miR-125b-5p levels are increased in a middle cerebral artery occlusion model and in an oxygen and glucose deprivation (OGD) model [35]. The authors found that the overexpression of miR-125b-5p reduced cystathionine β-synthase expression and hydrogen sulfide generation and thus alleviated cerebral OGD injury [35]. In addition, miR-125b-5p can inhibit angiogenesis through the translational suppression of vascular endothelial-cadherin in endothelial cells [36]. Angiogenesis during the formation of new brain microvessels is a crucial factor for recovery from stroke [37]. Moreover, miR-125b-5p is involved in inflammation-associated processes; for example, miR-125b-5p potentiates the activated nature of macrophages and their functional role in inducing immune responses [38] and regulates TNF-α expression in neonatal monocytes with altered proinflammatory reactions [39].

Although no published reports have previously assessed miR-192-5p levels after AIS, its role in acute ischaemia and reperfusion (I/R) injury in other tissues and organs has been highlighted recently in both patients and animal models. For example, serum levels of miR-192-5p are elevated after I/R-induced liver injury, and they correlate with the degree of liver damage [40]; urinary levels of miR-192-5p are increased after I/R-induced kidney injury and can serve as potential diagnostic markers [41]; and miR-192-5p levels are significantly increased in ischaemic muscles, which is related to its effect on anti-proliferative and proapoptotic activity [42]. Considering the previous relevant studies and the results of this study, all data indicate that miR-192-5p plays a role in stroke rather than simple acute behaviours after AIS, although these specific biological values still need to be clarified.

Our study had some limitations. First, although the results were significant, the sample size was small. Therefore, further studies using sequencing in all cohort or with a larger sample are necessary to confirm our pilot findings. Additionally, we need to improve the follow-up

rate as 10 patients in our study were lost during follow-up, which may lead to bias. Second, serial measurements of plasma miRNAs were not well conducted in our study. Specifically, miRNAs were measured in a small number of samples collected before thrombolysis (32 of 84 patients). In addition, the variation of onset-to-thrombolysis time need to be considered, for the expression of miRNAs may be changed with time after onset. Additionally, taking measurements at multiple time points within the first 24 h and during the follow-up period may better identify possible changes. Third, accurate assessment of infarct volume is more conducive to the prediction of prognosis; we could not get infarct volume on the same time period. Future studies need to consider this issue. Fourth, miR-124-3p, miR-125b-5p and miR-192-5p are expressed in various tissues, but not only in the brain [36,38,40,43]. These miRNAs can be released into the circulation for the disruption of the blood-brain barrier, or they can be carried by exosomes serving as signalling molecules that cross the blood-brain barrier [16]. In addition, the non-cerebral pathology associated with stroke-induced systemic reactions may also contribute to the alteration of miRNAs in the circulation. Thus, the precise sources and mechanisms underlying the observed differences in miRNAs remain to be determined.

In conclusion, recanalization by intravenous thrombolysis with or without endovascular treatment has become the most effective strategy for AIS in clinical practice worldwide [3,28]. To the best of our knowledge, reliable factors to predict neurological function deterioration and prognoses in AIS patients receiving recanalization are lacking. For the first time, our preliminary data showed that increased plasma levels of miR-124-3p, miR-125b-5p and miR-192-5p are associated with functional outcomes and stroke severity in AIS patients receiving thrombolysis. The present study provides supportive evidence for the involvement of the abovementioned miRNAs in AIS patients receiving thrombolysis, but further studies are needed to elucidate the biological mechanisms and clinical significance of these miRNAs.

Conflicts of interest

The authors declare that they do not have anything to disclose regarding conflicts of interest with respect to this manuscript.

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Author contributions

JRL and JJS conceived and designed the experiments; XWH, YHS, YSL and GFL performed the experiments; XWH, YHS, YSL, GFL, MTZ, RZ, YH and CCL helped with the specimen collection; XWH, YHS, YSL and JRL analysed and interpreted the data; XWH, YHS and YSL wrote the paper; JRL and JJS revised the paper. All authors approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.08.002.

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