

Original Article

Circulating microRNAs act as fingerprints in patients after acute aneurysmal subarachnoid hemorrhage

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Abstract: Aneurysmal subarachnoid hemorrhage (aSAH) is a highly morbid and fatal condition with high rate of cognitive impairment and negative impact in quality of life among survivors. Delayed cerebral infarction (DCI) is one the major factors for these negative outcomes. In this study we compared the circulating microRNA profiles at different periods of SAH patients. Peripheral blood samples on three different stages (after the onset of SAH, the peak period of cerebral vasospasm and cerebral vasospasm dissipation phase) of three patients were subjected to microarray analysis with Affymetrix miRNA 3.0 array and quantitative PCR analysis. 2549 miRNAs were detectable in the plasma of the 9 sample with the microarray technique. Three candidate miRNAs including hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 presented a significant expression with a higher expression in phase II while could be decreased in phase III of SAH. The areas under the receiver operating characteristic curves (AUC) of the validated three-miRNA signature were 0.702, 0.811 and 0.784 in predict phase I to II while the AUC for predict phase II to III were 0.703, 0.587 and 0.651, respectively. Combination of hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 possessed a moderate ability to discrimination phase I to Phase II and Phase II to Phase III with an area under ROC value of 0.976 and 0.902. In conclusion, our study demonstrated that hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 showed an up-regulation meanwhile through self-comparison, these data provide evidence that circulating miRNAs have the potential to be sensitive, cost-effective biomarkers for the dynamic monitoring the development of SAH.

Keywords: SAH, DCI, circulating miRNA, ROC, signature

Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) is a devastating illness that strikes healthy individuals during active and productive years of their life and results in significant death and disability [1, 2]. Vasospasm is a significant cause of morbidity and mortality after subarachnoid hemorrhage (SAH). Angiographic vasospasm is detected in 50 to 70% of patients with SAH, and delayed cerebral ischemia (DCI) secondary to vasospasm occurs in 19 to 46% of SAH patients and 54% of patients with poor grade SAH [3, 4]. The exact mechanisms of DCI have not been fully elucidated, although recent evidence has linked metabolic regulators of cerebral microvascular blood flow as important mediators in its development [4, 5]. In addition, the biomarker for monitoring the recovery phase of SAH still remains unclear.

The microRNAs (miRNA) are single-stranded RNA molecules of approximately 21 to 23 nucleotides in length, and they regulate gene expression at the posttranscriptional level by either degradation or translational repression of target mRNAs [6, 7]. Although miRNAs control a number of physiological conditions and diseases, only a few studies have shown the implications of miRNA in neuronal death and degeneration [8]. For example, compared to healthy control, miR-132 and miR-324 showed an upregulation in both SAH DCI and Non-DCI groups [1, 9, 10]. Another study regarding the biomarker for SAH indicated that t miR-502-5p and miR-1297 might be potentially valuable indicators of the diagnosis, severity and prognosis of SAH, and miR-4320 was a potentially valuable indicator of the diagnosis of SAH [11]. However, how to monitor the development of SAH in a dynamic condition or which miRNA

could be the fingerprint for the development of SAH still remain known.

In this study, we compared the circulating microRNA profiles of SAH patients and the circulating microRNA profiles of SAH patients during different stages including SAH patients after the onset (Phase I), the peak period of cerebral vasospasm (Phase II) and cerebral vasospasm dissipation phase (Phase III). Three candidate miRNAs was selected with the validation in a larger sample size. We further calculated the diagnosis potential for monitoring development of SAH in a dynamic condition through the ROC analysis.

Materials and methods

Patient recruitment

This study was approved by the local human ethics committees, and informed consent was obtained from all participants or their first-degree relatives for unconscious patients. The study was carried out in agreement with the Declaration of Helsinki. All of the patients were diagnosed in Jiangyin People's Hospital (Jiangyin, Jiangsu, China) between March 2014 and August 2016. Ruptured aneurysms were from patients presented with SAH, and the final diagnosis was made by digital subtraction angiography (DSA) or CTA examination. The location and type of SAH patients were determined by DSA and or CTA. To exclude a possible interference caused by DSA and/or treatment, all of the samples from ruptured patients were taken before DSA, and the pharmacological or procedural treatments if applicable. Smoking history was defined as regular tobacco consumption for > 1 year at the time of blood withdrawal. Criteria for positive clinical histories were hypertension, systolic blood pressure (BP) \geq 140 mmHg and/or diastolic BP \geq 90 mmHg; diabetes mellitus, fasting blood glucose \geq 7.0 mmol/L; and hypercholesterolemia, total cholesterol \geq 5.72 mmol/L and/or LDL-C \geq 3.64 mmol/L. To exclude a possible interference caused by hypertension, diabetes mellitus and sex, we only choose 9 samples from 3 patients according the blood flow velocity on transcranial Doppler sonography (TCD). All patients involved in the microarray study were free of other critical illness such as diabetes mellitus or hypertension disease.

Sample processing

Peripheral venous blood was collected into EDTA-containing tubes and processed within 120 minutes at the day 1, 3, 5, 7, 10, 14 and 21 after inpatient. Blood samples were processed as described previously [7, 12]. Whole blood was first centrifuged at 1600 g for 10 minutes, and then the supernatant was transferred into a fresh tube and centrifuged again at 9600 g for 10 minutes. The clear plasma was stored at -80°C until use. All procedures were carried out on dry ice.

RNA extraction and purification

Total RNA was extracted and purified using mirVana™ PARIS™ (Cat #AM1556, Ambion, Austin, TX, US), following the manufacturer's instructions to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US).

RNA labeling

MiRNA molecular in total RNA was labeled by miRNA Complete Labeling and Hyb Kit (Cat #5190-0456, Agilent technologies, Santa Clara, CA, US) followed the manufacturer's instructions, labeling section.

Array hybridization

Each slide was hybridized with 100 ng Cy3-labeled RNA using miRNA Complete Labeling and Hyb Kit (Cat #5190-0456, Agilent technologies, Santa Clara, CA, US) in hybridization Oven (Cat #G2545A, Agilent technologies, Santa Clara, CA, US) at 55°C, 20 rpm for 20 hours according to the manufacturer's instructions, hybridization section. After hybridization, slides were washed in staining dishes (Cat #121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat #5188-5327, Agilent technologies, Santa Clara, CA, US).

Data acquisition

Slides were scanned by Agilent Microarray Scanner (Cat #G2565CA, Agilent technologies, Santa Clara, CA, US) and Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US) with default settings. Raw data were normalized by Quantile algorithm, includ-

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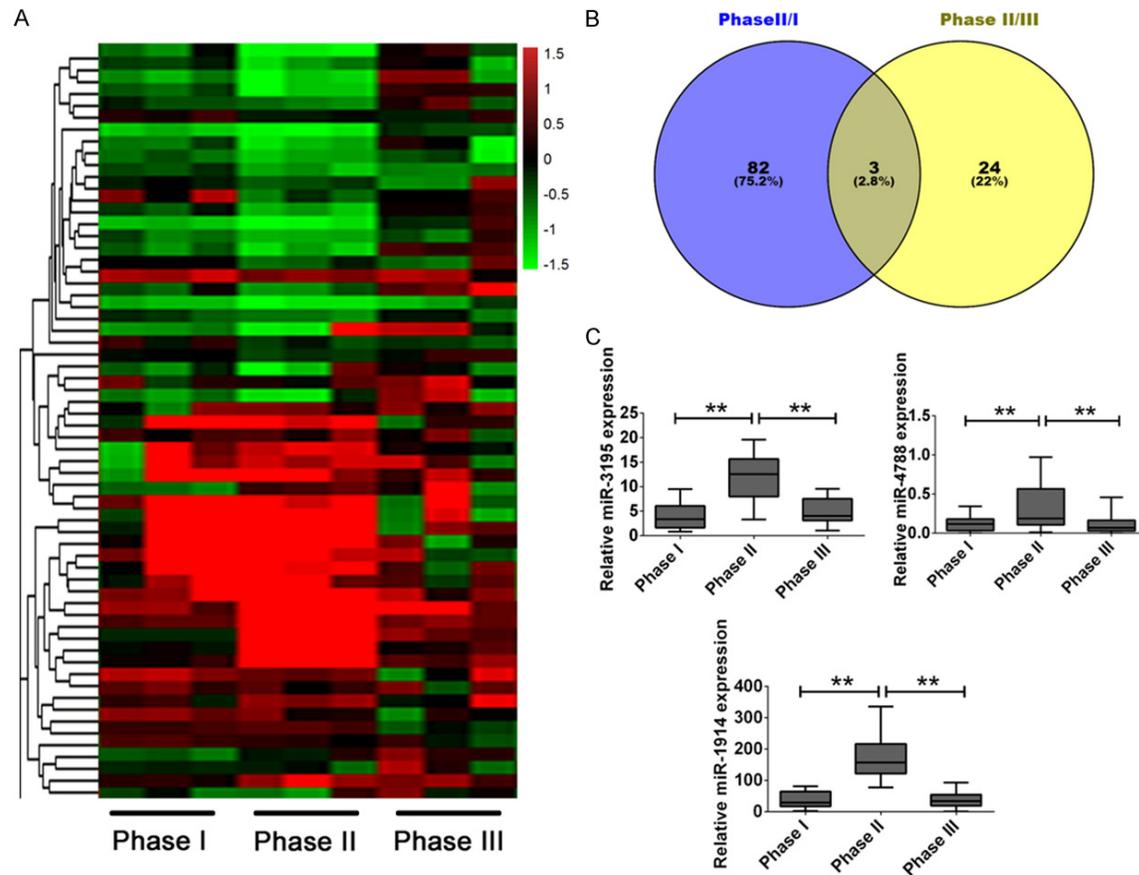


Figure 1. The hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 were confirmed as the candidate miRNAs according to the RT-PCR-based microarray. A: Cluster analysis of the different expression of the miRNAs. Phase I indicated the time after the onset of SAH, phase II indicated the peak period of cerebral vasospasm and phase III indicated cerebral vasospasm dissipation phase. B: Venny analysis to screen the potential candidate miRNA in three groups. C: Increased level of hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 was confirmed by RT-PCR in groups. Data were presented as box plot of the median and range of log-transformed relative expression level. The top and bottom of the box represent the seventy-fifth and twenty-fifth percentile. **indicated $P < 0.01$.

ed in the R package AgiMicroRna (López-Romero, P. Statistical Analysis BMC Genomic, 2011). The miRNA data were expressed as the median (interquartile interval), and other variables were expressed as the mean (SD). Chi-square test analysis of variance was used to evaluate statistical differences in demographic and clinical characteristics. The nonparametric Mann-Whitney U-test was used to compare differences in serum miRNA expression which are presented as box plot of the median and range of log-transformed relative expression level. The hierarchical cluster analysis (average linkage) was applied by using Cluster software and Treview. Risk score analysis was performed to investigate the effectiveness of the three-serum miRNA signature for SAH predict-

ing. Frequency tables and ROC curves were then used to evaluate the diagnostic effect of the profiling, to find the appropriate cutoff point, and to validate the procedure and cutoffs in the next validation sample set. ROC curves were then used to evaluate the diagnostic effects of the profiling and to find the appropriate cutoff point, and to validate the procedure and cutoffs in the next validation sample set. First, the risk score of each serum sample in the training set was calculated, as the basis of their risk scores and a set cut off, serum samples were then divided into a high-risk group, representing the possible SAH, and a low-risk group. At the optimal cutoff value with the value of sensitivity + specificity considered to be maximal. Statistical analysis was per-

formed using STATA 9.2, and presented with GraphPad Prism 5.0 software. Results were considered statistically significant at $P < 0.05$.

Results

Selection and validation phase

In order to gain an expression profile of circulating miRNAs that is specific for monitoring the development of SAH, the miRNAs microarray was used to identify the differentially expressed miRNAs in three SAH patients at three different phase including SAH patients after the onset, the peak period of cerebral vasospasm and cerebral vasospasm dissipation phase. A total of 2549 miRNAs were detectable in the plasma of the 9 sample pools with the microarray technique. The concentration of every sample on different stages was various, simultaneous upregulated or downregulated miRNA were found through paired-comparison. As presented in **Figure 1A**, the different expression of miRNAs was presented in different phase of different patients. According to the pathological development of SAH, we selected three candidate miRNAs by using the following filter condition: 1) Increased in peak period of cerebral vasospasm compared with SAH patients after the onset; 2) Decreased in cerebral vasospasm dissipation phase compared with the peak period of cerebral vasospasm; 3) The threshold that at most 30 of CT value and at least 4 average fold change in both two group for further individual validation.

As presented in **Figure 1B**, we further examined the three candidate miRNAs by RT-qPCR in a training sample set (20 patients in three different stages) and in a validation set (50 patients in three different stages). We found that all the three candidate miRNAs including hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 presented a significant expression with a higher expression in Phase II while could be decreased in Phase III of SAH (**Figure 1C**).

Risk score and ROC curve analysis

Since miRNAs has been applied for diagnosis biomarker of SAH, in this study, we supposed that whether the miRNA could be used as the fingerprints for monitoring the development of SAH. To assess the diagnostic value of the three-circulating miRNAs profiling system, we

used a risk score formula to calculate the risk score function in SAH samples with different phases. ROC curves analyses were conducted to assess the diagnostic sensitivity and specificity of the three miRNAs signature for SAH from Phase I to Phase II by using risk score functions (RSFs), The areas under the curve (AUC) were 0.702 (95% confidence interval (CI), 0.570-0.834), 0.811 (95% CI, 0.691-0.931), 0.784 (95% CI, 0.662-0.906) and 0.976 (95% CI, 0.933-1.019) for the samples in validation sets (**Figure 2A**), while for SAH from Phase II to Phase III, The AUC were 0.703 (95% confidence interval (CI), 0.569-0.837), 0.587 (95% CI, 0.444-0.730), 0.651 (95% CI, 0.511-0.791) and 0.902 (95% CI, 0.819-0.985) for the samples in validation sets (**Figure 2B**). The results indicated that the three-miRNA signature can serve as a novel non-invasive approach for the development screening of SAH from Phase I to Phase III.

Discussions

Delayed neurologic deterioration is assumed to be due to vasospasm-induced ischemia. Nevertheless, there is growing pathophysiological evidence that multiple factors other than cerebral vasospasm contribute to poor outcomes, and the contribution of these processes to outcomes might be greater than that of vasospasm [13-15]. These include delayed neuronal apoptosis, early brain injury, reactive oxygen species and other free radicals, inflammation, microcirculatory vasospasm, microthromboembolism, and cortical spreading depolarization [16, 17]. Although miRNAs control a number of physiological conditions and diseases, but few studies up to date related to circulating miRNA profiling about DIND.

Differential miRNA expression in SAH patients reflects the result of hemorrhage. We demonstrated 2549 miRNAs were detectable in the plasma at least in 1 of the 9 sample pools with the microarray technique. The hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 showed a upregulation meanwhile through self-comparison among three SAH DCI groups. These identified pathways also aligned with the fact that SAH patients might develop delayed cerebral infarction, which involved ischemic injury. The major finding in this study is that, compared with healthy controls. In addition, the risk score analysis revealed that hsa-miR-3195,

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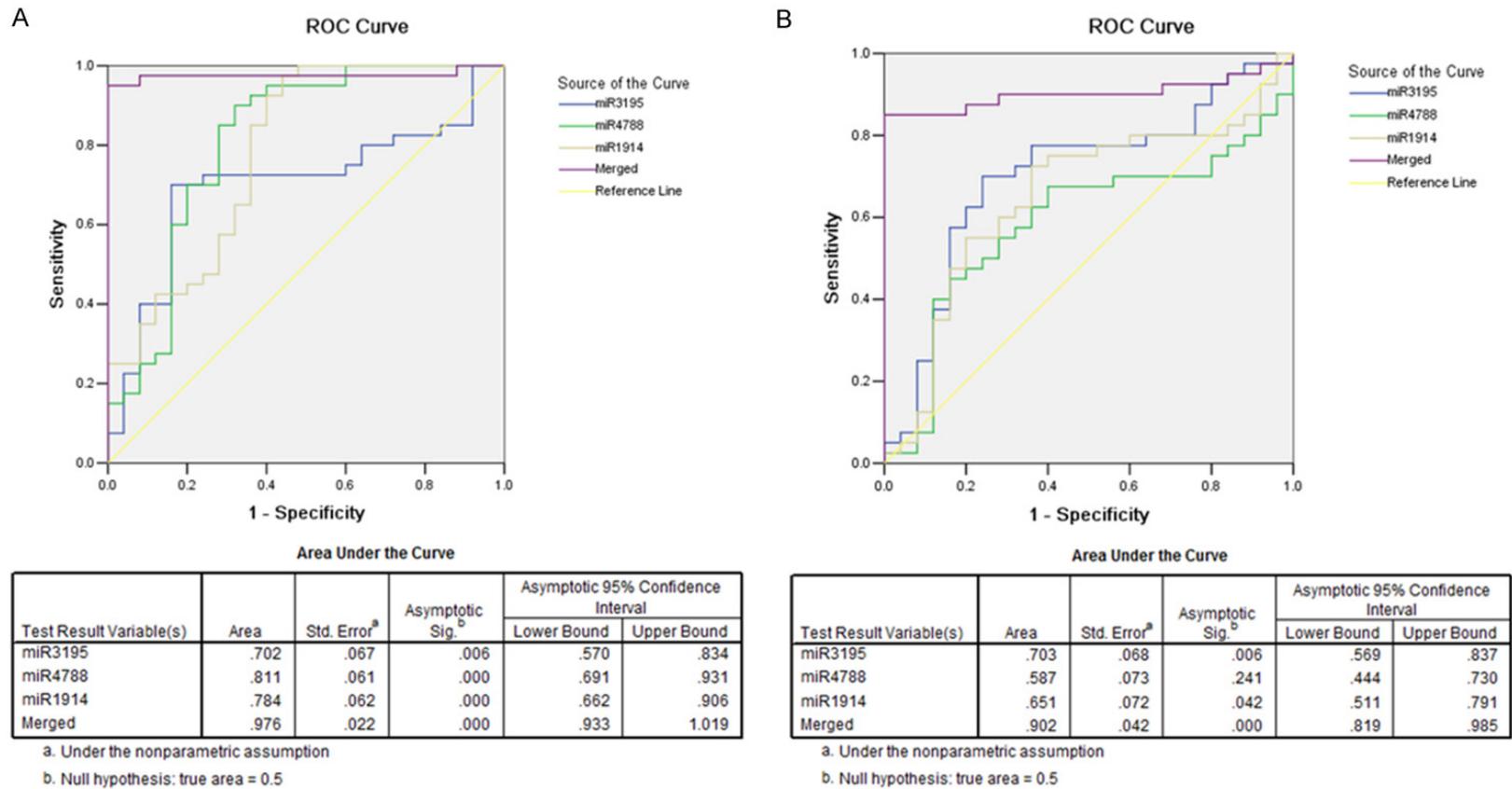


Figure 2. ROC analysis of the three potential biomarkers for SAH. A: ROC curve analysis was conducted for discrimination between SAH patients in phase I and phase II. B: ROC curve analysis was conducted for discrimination between SAH patients in phase II and phase III.

hsa-miR-4788 and hsa-miR-1914 could distinguish the SAH patients in phase I from phase II and SAH patients in phase II from phase III, indicating that the three-miRNA signature can serve as a novel non-invasive approach for the development screening of SAH from Phase I to Phase III.

Because these miRNAs were unanimously changed in both ruptured and unruptured cases, it was unlikely that the observed changes in circulating miRNA were caused by secondary complications of IA, such as SAH and neural damages [18, 19]. Moreover, all of the samples from unruptured patients were taken before any pharmacological and/or surgical treatments; hence, it is also unlikely that the altered circulating miRNA levels in IA patients were a result of clinical treatments [20]. Results from previous genomewide studies on IAs indicated that the data reproducibility across different microarray experiments were relatively poor.

In conclusion, our study demonstrated that hsa-miR-3195, hsa-miR-4788 and hsa-miR-1914 showed a upregulation meanwhile through self-comparison, these data provide evidence that circulating miRNAs have the potential to be sensitive, cost-effective biomarkers for the dynamic monitoring the development of SAH.

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Disclosure of conflict of interest

None.

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