The Diagnostic Value of Circulating Brain-specific MicroRNAs for Ischemic Stroke

Zhong-Bao Yang1,2, Ting-Bo Li2, Zhen Zhang3, Kai-Di Ren2, Zhao-Feng Zheng1, Jun Peng2,4 and Xiu-Ju Luo1

Abstract

Objective  Circulating microRNAs have been recognized as promising biomarkers for various diseases. The aim of the present study was to explore the potential role of circulating miR-107, miR-128b and miR-153 as non-invasive biomarkers in the diagnosis of ischemia stroke.

Methods  One hundred and fourteen ischemic stroke patients (61±11.3 years old) and 58 healthy volunteers (56±3.9 years old) matched for age and sex were enrolled in this study. Total RNA was isolated from plasma with TRIzol reagent. The circulating microRNAs levels were measured by quantitative real-time polymerase chain reaction.

Results  The circulating levels of miR-107, miR-128b and miR-153 significantly increased 2.78-, 2.13- and 1.83-fold in ischemia stroke patients in comparison to the healthy volunteers, respectively. Receiver operating characteristic (ROC) curves were analyzed using the SPSS software program and revealed the areas under the curve for circulating miR-107, miR-128b and miR-153 to be 0.97, 0.903 and 0.893 in ischemia stroke patients in comparison to healthy volunteers, respectively. The levels of circulating miR-107, miR-128b and miR-153 therefore positively correlated with the severity of stroke as defined by NIHSS classes.

Conclusion  Our results suggest that circulating miR-107, miR-128b and miR-153 might be used as potential novel non-invasive biomarkers for the diagnosis of ischemia stroke. However, future prospective trials in large-sized patient cohorts are needed before drawing any definitive conclusions.

Key words: circulating miRNAs, ischemia stroke, biomarkers, diagnosis


Introduction

Stroke, including ischemic and haemorrhagic stroke, is one of the leading causes for death and permanent disability worldwide (1). Most strokes (about 80%) are ischemic strokes caused by an interruption of the blood supply to the brain. Currently, the diagnosis of stroke mainly relies on clinical examination and imaging techniques (such as computed tomography scans-CT and magnetic resonance imaging-MRI). There are no specific plasma biomarkers for the clinical diagnosis of stroke yet. Although imaging-based techniques (CT or MRI) are relatively sensitive and specific, small infarcts are still difficult to detect with computed tomography and an infarct may not be detected by conventional (T1/T2) MRI within 6 hours from onset (2). Moreover, these diagnostic modalities are costly and inconvenient, especially for remote and poor areas. As a result, there is a clinical demand for specific and reliable non-invasive biomarkers for supporting clinical diagnosis, classification and prognosis of stroke. RNA-based studies have demonstrated as promising biomarker candidates for stroke (3-5). For example, blood mRNA profiles have been suggested to be potential genomic biomarkers for ischemic stroke (6). However, research on these biomarkers is still at an elementary stage, and they are not yet used in clinical practice.
MicroRNAs (miRNAs) are a class of small (-22 nucleotides long), single stranded endogenous non-coding RNAs that bind to the 3’-untranslated region of target genes to downregulate their expression (7). It is well established that miRNAs play important roles in various physiological and pathological processes, such as regulating cell differentiation, proliferation, inflammation and apoptosis (8,9). MiRNAs are also present in plasma or serum in the forms of microvesicles and/or protein-miRNA complexes, the so-called circulating miRNAs, which are stable and reproducible (10-12). Over the past few years, it has been reported that plasma miRNAs are sensitive and specific biomarkers for various pathological conditions including tissue injury, as they reflect the events that occur at the site of injury (13-15). To date, hundreds of miRNAs have been reported and some of them are specifically expressed or greatly enriched in certain tissues, and thus they are called tissue-specific or tissue-enriched miRNAs (16). By analyzing current available literature, we identified 4 miRNAs (miR-107, miR-128b, miR-153 and miR-137) that had been reported to be brain-specific or brain-enriched. However, it is not known whether these miRNAs can be detected in the plasma and whether or not they demonstrate significant changes in the peripheral blood of patients suffering ischemic stroke. In this study, we detected the plasma levels of miR-107, miR-128b, miR-153 and miR-137 in patients suffering ischemic stroke and in healthy volunteers, and explored their potential value as biomarkers in the diagnosis of ischemic stroke.

Materials and Methods

Study Subjects

Patients with acute ischemic stroke (n=114) and healthy volunteers (n=58) from Xiangya Hospital (Changsha, China) were enrolled. All patients were newly diagnosed based on the results of neurological and computed tomography (CT) or magnetic resonance imaging (MRI) examinations. Patients with the following diseases, including embolic brain infarction, transient ischemic attack, cerebrovascular malformation and subarachnoid haemorrhage, brain tumor and severe systemic diseases (such as collagenosis, liver or renal diseases), were excluded. Age- and sex-matched controls, free of any neurological diseases based on the same exclusion criteria as mentioned above (n=58), were selected from community-based inhabitants. The relevant characteristics of stroke patients and controls are summarized in Table 1. The stroke subtype was sorted through the Oxfordshire Community Stroke Project (OCSP). The stroke severities were evaluated according to the National Institutes of Health Stroke Scale (NIHSS) on admission.

This study has been conducted in conformity with the Declaration of Helsinki (2013) of the World Medical Association, and was approved by the local Ethics Committee at Xiangya Hospital of Central South University (Changsha, China). Written informed consent was obtained from all individuals at the time of enrollment. All investigators were qualified to undertake this study.

Samples Collection

Peripheral venous blood samples (3 mL) were obtained from the enrolled patients within 24 hours of hospital admission, whereas the blood samples from the controls were taken before their breakfast. Plasma specimens were isolated by two steps of centrifugation. First, the samples were centrifuged at 1,500× g for 10 minutes at 4°C for the collection of supernatants followed by centrifuging again at 14,000× g for 15 minutes at 4°C for the collection of pure plasma, which was stored in RNase-free tubes at -80°C until the measurements.

RNA Isolation from Plasma

Total RNA was isolated by using TRIzol reagent according to manufacturer’s instructions (TakaRa, Dalian, China). The concentration and purity of RNA were determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The RNA samples were stored at -80°C for future use.

Detection and Analysis of miRNAs by qRT-PCR

MiRNAs expressions were detected by real-time quantita-

Table 1. Baseline Characteristics of Ischemic Stroke Patients and Control Subjects.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>Stroke</th>
<th>X²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total subject, n</td>
<td>58</td>
<td>114</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>35(60.3)</td>
<td>78(68.4)</td>
<td>0.462</td>
<td>0.387</td>
</tr>
<tr>
<td>Age, y (mean±SD)</td>
<td>56±3.9</td>
<td>61±11.3</td>
<td>—</td>
<td>0.041</td>
</tr>
<tr>
<td>Cholesterol, mmol/L (mean±SEM)</td>
<td>4.75±0.14</td>
<td>4.98±0.13</td>
<td>—</td>
<td>0.034</td>
</tr>
<tr>
<td>Triglyceride, mmol/L (mean±SEM)</td>
<td>1.86±0.12</td>
<td>1.76±0.14</td>
<td>—</td>
<td>0.623</td>
</tr>
<tr>
<td>HDL-C, mmol/L (mean±SEM)</td>
<td>1.53±0.02</td>
<td>1.30±0.06</td>
<td>—</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C, mmol/L (mean±SEM)</td>
<td>2.78±0.81</td>
<td>2.79±0.78</td>
<td>—</td>
<td>0.876</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>9(15.5)</td>
<td>92(80.7)</td>
<td>48.8</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>4(6.80)</td>
<td>37(32.5)</td>
<td>18.6</td>
<td>&lt;0.00</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>10(17.2)</td>
<td>25(21.9)</td>
<td>0.075</td>
<td>0.772</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>17(29.3)</td>
<td>49(43.0)</td>
<td>17.9</td>
<td>&lt;0.00</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD or n (%). p value was calculated by ANOVA or χ²test.
Figure 1. Plasma expression levels of miR-107, miR-128b, miR-153 and miR-137 in stroke patients and healthy volunteers. *p<0.01

Biochemical and clinical assays

Fasting blood sugar (FBS), triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) were measured by an automatic analyzer (Hitachi75, Tokyo, Japan). The clinical history, family history, drug history and physical examination findings were recorded.

Statistical analysis

The statistical analysis was performed using the SPSS software program (version 20). Data were expressed as the means ±S.E.M. Differences among the multiple groups were analyzed by ANOVA with Bonferroni’s multiple comparison tests. For categorical variables, the Chi-Square ($\chi^2$) test was used. The ROC analysis was used to evaluate the diagnostic accuracy of each circulating miRNA for all the groups. The area under the ROC curve (AUC) was considered to be a diagnostic index. All tests were 2-sided and a significance level of p<0.05 was considered to be statistically significant.

Results

The expression levels of circulating MiRNAs in ischemic stroke patients versus healthy volunteers

As shown in Fig. 1, the levels of plasma miR-107, miR-128b and miR-153 in stroke patients were 2.78-fold (2.78±0.10), 1.83-fold (1.83±0.13) and 2.13-fold (2.13±0.10) higher than those in healthy volunteers, respectively, which
were significantly increased in pairwise comparisons between the stroke patients and controls (p<0.01). However, there was no significant difference in the plasma level of miR-137 between the stroke patients and healthy controls (p >0.05). Therefore, we mainly focused on miR-107, miR-128b and miR-153 in the subsequent analysis.

According to the standard of OCSP, ischemic stroke was classified as total anterior circulation infarction (TACI), partial anterior circulation infarction (PACI), lacunar circulation infarction (LACI) or posterior circulation infarction (POCI). As displayed in Supplementary Table 1, there were no significant differences in the levels of miRNAs among the stroke subtypes. Since the age and smoker ratio between the healthy volunteers and patients did not fully match in this study, we therefore analyzed the impact of the age and smoking on the levels of miRNAs. We did not find any close correlation between the age and the levels of miRNAs (Supplementary Figure 1, 2). After excluding the smokers from the healthy volunteers and patients, miR-107, miR-128 b and miR-153 were still up-regulated in non-smoking stroke patients compared to the non-smoking volunteers, whereas there was still no significant change in miR-137 (Supplementary Table 2).

Potential Role of Plasma miR-107, miR-128b or miR-153 in Stroke Diagnosis

To examine the potential role of miR-107, miR-128b or miR-153 in the stroke diagnosis, a ROC analysis was performed for all recruited subjects. The ROC curves of plasma miR-107, miR-128b and miR-153 demonstrated remarkable differences between the healthy volunteers and the stroke patients, with an area under curve (AUC) of 0.971, 0.881 and 0.738, respectively (Fig. 2). These results indicated that plasma miR-107, miR-128b and miR-153 might be valuable biomarkers for stroke patients. Additionally, the AUC, maximum cut-off point, sensitivity, specificity, 95% CI (confidence interval) and p value for miR-107, miR-128b and miR-153 were briefly summarized in Table 2.

Correlations of miR-107, miR-128b and miR-153 with NIHSS Score

The correlation analysis between miRNA levels and NIHSS score was done using Pearson’s correlation coefficients. The levels of circulating miRNAs increased in parallel to the increased severity of stroke as defined by NIHSS classes (r=0.225, p=0.002) (Fig. 3).

Discussion

Since an ideal biomarker of tissue injury should be abundant and preferentially (or exclusively) produced in the tissue of interest, we thus focused on brain-specific or -enriched miRNAs. In the present study, we observed that the plasma levels of brain-specific or brain-enriched miR-107, miR-128b and miR-153 in stroke patients were significantly increased in comparison to those in healthy controls and that the elevations of these miRNAs were positively correlated with the stroke severities as assessed by the NIHSS score. Furthermore, we evaluated the diagnostic value of plasma miR-107, miR-128b and miR-153 by the ROC curve. We found that AUC values of miR-107, miR-128b and miR-153 were significantly increased in stroke patients in comparison to those in the healthy controls. Among the three miRNAs, the increase of miR-107 was the most obvious and showed the highest sensitivity and specificity. In a rat ischemic stroke model, the expressions of miR-107, miR-128b and miR-153 in brain tissues were significantly up-regulated compared with those in the controls, which closely correlated with their changes in plasma observed in stroke patients (9). These results strongly indicated that plasma miR-107, miR-128b and miR-153 might be useful as potential non-invasive biomarkers for the diagnosis of ischemic stroke. Although some characteristics (such as age and smoker ratio) did not fully match between the healthy controls and stroke patients, we did not see any close association of circulating miRNAs levels with age or smoking, which ruled out the impact of age or smoking on circulating miRNAs in this study. In addition, we compared the circulating miRNAs levels among stroke subtypes, and no significant differences were observed, suggesting that these miRNAs (miR-107, miR-128b and miR-153) are not potential candidates of biomarker for differential diagnosis of stroke subtypes.

Among the 4 miRNAs (miR-107, miR-128b, miR-153 and miR-137) screened in the present study, circulating levels of miR-128b and miR-153 were the first time to be examined in ischemic stroke patients, whereas miR-107 and miR-137 were reported in similar studies before (17, 18). In a study by Duan et al., they did not observe a significant change in the plasma level of miR-107 in ischemic stroke patients (18), whereas we did. In another study, Zhao et al. found a decreased level of miR-137 in post-stroke depression rats (17), but we did not see any obvious change of miR-137 in this study. The reasons for these discrepancies in same miRNA levels among the different studies are not clear. The differences in species (rat, mouse or human), process of stroke (acute or chronic phase) or methods for miRNAs measurement (miRNA array or real-time PCR) might account, at least in part, for the discrepancies among the different studies.

To date, the diagnosis of ischemic stroke mainly depends on image techniques, such as CT and MRI. A desirable blood marker for ischemic stroke, however, is still lacking. Some ischemic brain injury-related proteins, such as calcium binding protein B (S-100B), neuron specific enolase (NSE), myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP) (19-22), have been suggested as additional diagnostic biomarkers for stroke. However, these proteins are not ideal biomarkers for ischemic stroke due to either a lack of specificity or a low sensitivity. Furthermore, the blood brain barrier restricts their release into the peripheral blood. Therefore, there is still a clinical need for a novel
Diagnostic accuracy of circulating miR-107, miR-128b and miR-153 were analyzed by ROC curve (stroke patients vs. healthy volunteers). A: ROC curve of miR-107. B: ROC curve of miR-128b. C: ROC curve of miR-153.

Table 2. Receiver Operator Characteristic Curve (ROC) Analysis of MiRNA Ratios in the Prediction of Stroke Patients.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>AUC</th>
<th>Cut-off point</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>95%CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-107</td>
<td>0.97</td>
<td>2.03</td>
<td>0.938</td>
<td>0.922</td>
<td>0.929-0.991</td>
<td>0.00</td>
</tr>
<tr>
<td>miR-128b</td>
<td>0.903</td>
<td>1.57</td>
<td>0.728</td>
<td>0.92</td>
<td>0.853-0.953</td>
<td>0.00</td>
</tr>
<tr>
<td>miR-153</td>
<td>0.893</td>
<td>1.1</td>
<td>0.912</td>
<td>0.74</td>
<td>0.837-0.950</td>
<td>0.00</td>
</tr>
</tbody>
</table>

AUC: Area under the curve, 95%CI (confidence interval)

Biomarker, which is able to reliably rule-in or rule-out ischemic stroke patients immediately upon admission. MiRNAs are abundantly expressed in nervous system and may release from the damaged cells, leading to increase in plasma levels of miRNAs (23). Emerging evidence suggests that the plasma miRNA profiles are stable and reproducible, which have been proposed as novel noninvasive biomarkers for certain diseases such as cancers and acute myocardial in-
fraction (14, 24, 25). In ischemic stroke, the destruction of blood brain barrier and brain tissue injury release miRNAs into circulating blood (23, 26-28). To a certain extent, the levels of circulating miRNAs, especially brain-specific or brain-enriched miRNAs, may reflect the severity of cerebral infarction (29, 30). In this study, our results showed that the plasma levels of the selected brain-specific or enriched miRNAs (miR-107, miR-128b and miR-153) were significantly elevated in stroke patients and they positively correlated with the severity of cerebral ischemic injury, thus confirming the diagnostic value of such miRNAs in ischemic stroke. It is worth mentioning that we did not find any significant difference in the plasma level of miR-137, thereby ruling out the possibility for miR-137 as a potential biomarker for ischemic stroke.

The pathophysiological significance for the changes in plasma levels of miR-107, miR-128b and miR-153 remains unclear. Using the Target Scan 6.2 software program and miRbase 19, we predicted a number of potential targets for miR-107, miR-128b or miR-153. Among these targets, some are involved in cerebral ischemic injury. For example, glutamate transporter-1 (GLT-1) is a potential target of miR-107 or miR-128b. GLT-1 plays a major role in the clearance of the glutamate released in the synaptic cleft to maintain glutamate homoeostasis in central nervous system and thus to prevent glutamate accumulation and excitatory neurotoxicity (31). There were reports that the GLT-1 expression was dramatically down-regulated in the brain following cerebral ischemia (32). However, the mechanisms responsible for such down-regulation remain largely unknown. Since GLT-1 is a potential target gene of miR-107 and/or miR-128b and these two miRNAs were elevated in the peripheral blood of stroke patients, we thus speculate that the down-regulation of GLT-1 expression after cerebral ischemia might be due to, at least in part, the increase in miR-107 and/or miR-128b expressions. The plasma levels of miR-107 and/or miR-128b might be served as biomarkers to reflect the status of excitatory neurotoxicity in ischemic stroke patients. As for miR-153, its predicted target proteins are calcium/calmodulin-dependent serine protein kinase (CASK), nuclear factor erythroid 2-related factor 2 (NRF2) and alpha-synuclein (SNCA), etc., which were reported to be involved in the pathogenesis of traumatic brain injury (33). It is unknown, however, whether the elevation of miR-153 in peripheral blood of stroke patients is associated with the alteration in such proteins.

It is worth pointing out that numerous circulating miRNAs were reported to have potential values in diagnosis of ischemic stroke, but no miRNAs have yet been clearly identified so far. Thus, at this moment, circulating miRNAs
including those reported in this or other studies are not sufficient to assist in making a differential diagnosis of ischemic stroke from healthy status or other diseases without imaging diagnosis. There is still a long way to go before identifying miRNAs with recognized clinical value in the diagnosis and/or prognosis of ischemic stroke.

**Study limitations**

There are several limitations that need to be acknowledged and addressed for this study. First of all, the sample size was relatively small and some characteristics (such as age and smoker ratio) were not well-matched between the healthy controls and stroke patients. Although we ruled out the impact of the age and smoking on the circulating miRNAs, we still could not rule out the interference of some other factors (such as hypertension and diabetes) in this study. Secondly, we only measured the circulating miRNAs at the acute phase of stroke, which compromised the clinical values for this study. For example, without the data at the chronic phase of stroke and the functional outcome, we were not able to evaluate the prognostic values of the circulating miRNAs for ischemic stroke. Finally, cut-off values for miRNAs seem to be overfitted to this cohort. Therefore, they should be tested in different cohorts in future studies.

**Conclusion**

In the present study, we demonstrated, for the first time, that the plasma levels of miR-107, miR-128b and miR-153 were dramatically elevated in ischemic stroke patients and the elevations were positively associated with the stroke severity. Therefore, the brain-specific or enriched miRNAs (such as miR-107, miR-128b and miR-153) might be a novel class of minimal invasive biomarkers for helping in the diagnosis of ischemic stroke and the severity evaluation. It may provide a basis for future large-scale studies that can validate our findings in the future.

**The authors state that they have no Conflict of Interest (COI).**

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