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RESEARCH

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Elevation of brain-enriched miRNAs in cerebrospinal fluid of patients with acute ischemic stroke

Sofie Sølvsten Sørensen^{1*}, Ann-Britt Nygaard², Anting Liu Carlsen³, Niels H. H. Heegaard³, Mads Bak⁴ and Thomas Christensen¹

Abstract

Background: The purpose of this study was to investigate the potential of cerebrospinal fluid miRNAs as diagnostic biomarkers of acute ischemic stroke using three different profiling techniques in order to identify and bypass any influence from technical variation.

Methods: Cerebrospinal fluid (CSF) from patients with acute ischemic stroke ($n = 21$) and controls ($n = 21$) was collected by lumbar puncture. miRNA analysis was performed with three different methods: 1) Trizol RNA extraction followed by Illumina Next Generation Sequencing (NGS) on all small RNAs, 2) Exiqon RNA extraction protocol and miRNA qPCR assays, and 3) validation of 24 selected miRNAs with Norgen Biotek RNA extraction protocol and Applied Biosystems qPCR assays.

Results: NGS detected 71 frequently expressed miRNAs in CSF of which brain-enriched miR-9-5p and miR-128-3p were significantly higher in CSF of stroke patients compared to controls. When dividing stroke patients into groups according to infarct size several brain-enriched miRNAs (miR-9-5p, miR-9-3p, miR-124-3p, and miR-128-3p) were elevated in patients with infarcts >2 cm³. This trend appeared in data from both NGS, qPCR (Exiqon), and qPCR (Applied Biosystems) but was only statistically significant in some of the measurement platforms.

Conclusions: Several brain-enriched miRNAs are elevated in the CSF three days after stroke onset, suggesting that these miRNAs reflect the brain damage caused by ischemia. The expression differences seem, however, limited to patients with larger ischemic brain injury, which argues against the use of CSF miRNAs as diagnostic biomarkers of stroke based on current methods.

Keywords: Stroke, MicroRNA, Biomarkers, Brain ischemia, Cerebrospinal fluid

Background

Ischemic stroke is a frequent cause of death and disability all over the world. In patients with atypical or discrete symptoms the diagnosis of ischemic stroke can be difficult and determining the cause of stroke in each individual is even more complicated. Despite thorough clinical and paraclinical evaluation comprising brain computed tomography (CT) or magnetic resonance imaging (MRI), blood tests, cardiac workup, and ultrasound examination of the carotid arteries, the cause of acute ischemic stroke

remains unknown in 25% of all patients [1]. A biomarker with the ability to distinguish between different stroke etiologies (large-artery atherosclerosis, small-artery occlusion, and cardio-embolism) could potentially aid the physicians in choosing the best secondary prevention for their patients since treatment of the different stroke subtypes is not the same. In patients with large-artery atherosclerosis or small artery occlusion, anti-platelet drugs are first choice treatment for reducing the risk of stroke recurrence [2]. In contrast, in patients with cardio-embolic stroke, anticoagulant therapy is the most effective prevention [3, 4]. Surgical intervention with carotid endarterectomy is considered in large-artery atherosclerosis as it reduces recurrent strokes in patients with a

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significant ipsilateral carotid artery stenosis [5]. The current and most widely used system for categorization of ischemic stroke subtypes, the TOAST classification, is based on clinical and paraclinical test results and has only moderate inter-rater reliability [6, 7]. Furthermore, many stroke patients have competing possible causes, such as concurrent atrial fibrillation and carotid artery stenosis, making it impossible to establish a specific etiology. Therefore, new diagnostic and prognostic biomarkers in relation to ischemic stroke are warranted and could potentially benefit stroke patients in various ways.

MicroRNAs (miRNAs) are small non-coding RNA molecules involved in numerous physiological processes in which they function as posttranscriptional inhibitors of gene expression. Their presence in different body fluids such as blood and CSF combined with their stability during long term storage make them interesting as biomarker candidates [8–10]. Recently, several clinical stroke studies have suggested a diagnostic value of miRNAs extracted from plasma/serum and circulating blood cells [11–15], although the results points in various directions. Since CSF is separated from the blood by the blood brain barrier (BBB) and because of its proximity to the brain tissue, it is obvious to assume that the CSF could contain unique signatures of miRNA expression specific for various central nervous system (CNS) pathologies, and therefore serve as a more valid biomarker source compared to other body fluids.

Within few minutes after stroke onset, various brain functions begin to break down as a direct consequence of ischemia including changes in gene expression [16, 17]. Microarray studies have identified several gene families, e.g. immediate early genes, heat shock protein genes, and genes related to apoptosis and inflammation that are regulated by focal brain ischemia [18–20]. Several experimental studies have shown changes in the expression of miRNAs in rat brain tissue following middle cerebral artery occlusion [21–26]. Based on this knowledge, we hypothesized that the changes in miRNA expression in the brain of acute ischemic stroke patients are reflected in the cerebrospinal fluid (CSF) surrounding the central nervous system.

Previously, we have explored the expression of miRNAs in CSF of acute stroke patients in a pilot study [27]. We found 21 frequently expressed miRNAs in the CSF of which two miRNAs (miR-221-3p and let-7c-5p) appeared to be up-regulated in stroke patients compared to controls, although not statistically significant after correction for multiple testing.

The purpose of this follow-up study was 1) to investigate the potential of CSF miRNAs as biomarkers of acute ischemic stroke using three different profiling techniques in order to identify and bypass any influence from technical variation and 2) to validate our previous findings in a larger group of patients.

Methods

Patient material and sample collection

Patients admitted with acute ischemic stroke at the Department of Neurology, Nordsjællands Hospital, University of Copenhagen, from June 2013 to December 2014 were asked to participate in accordance with the approval from the Danish Research Ethics Committee (project ID: H-I-2011-094).

As in our previous study, the stroke diagnoses were based on patient history, thorough neurologic examination and CT scan of the brain on admission. In addition, all patients were scanned on a 3 Tesla System Philips Achieva MRI scanner on the third day after symptom onset. The imaging protocol consisted of diffusion-weighted and T₂-weighted sequences. Infarct volumes were calculated from MR diffusion-weighted images by multiplying the summed areas of infarction with the distance between each section [28]. After additional Doppler Ultrasound of carotid arteries and cardiac telemetry for at least 48 h during admission patients were categorized according to stroke subtype using the TOAST classification [6]. Controls were recruited from our outpatient neurology clinic as they underwent planned lumbar punctures as part of their investigation program for neurologic diseases others than stroke. A total of 42 patients were included, $n = 21$ ischemic stroke patients, and $n = 21$ controls. Patient characteristics are given in Table 1. A more detailed description of the different diagnoses and stroke parameters in each individual patient is given in Additional file 1. The

Table 1 Patient characteristics

Characteristics	Stroke ($n = 21$) Mean (range) or n (%)	Control ($n = 21$) Mean (range) or n (%)
Age (years)	66.6 (47–78)	66.0 (31–86)
Male sex	12 (57.1)	13 (61.9)
NIHSS	2.5 (0–10)	-
Infarct (cm ³)	6.7 (0.3–32.4)	-
SAO	11 (52.4)	-
LAA	5 (23.8)	-
CE	2 (9.5)	-
U	3 (14.3)	-
Dementia	-	7 (33.3)
MCI	-	2 (9.5)
Multiple sclerosis	-	1 (4.8)
ALS	-	1 (4.8)
No CNS disease	-	10 (47.6)

NIHSS National Institute of Health Stroke Scale, SAO stroke caused by small-artery occlusion, LAA, stroke caused by large-artery atherosclerosis, CE stroke caused by cardioembolism, U stroke of undetermined etiology, MCI mild cognitive impairment, ALS amyotrophic lateral sclerosis, No CNS disease patients with no evidence of disease in the central nervous system (benign headache, Bell's palsy, polyneuropathy, restless legs or lower back pain)

composition of diagnoses and severity of stroke symptoms were comparable to our previous study, Additional file 2.

Lumbar punctures were done by sterile technique on the third day after stroke onset with the use of a 0.7 mm spinal needle in accordance with local guidelines. At least 5 ml CSF were collected from each patient. For miRNA detection 1 ml CSF from each patient was centrifuged at 2000 x g at 4 °C for 15 min immediately after collection and the cell-free fractions were stored at -80.0 °C. Analyses of miRNAs were performed by three different methods as described below. A detailed description of the methodology is available online, Additional file 3.

Trizol RNA extraction followed by Illumina next generation sequencing

RNA was isolated from 100 µl CSF using TriZol reagent (Invitrogen) according to the manufacturer's protocol. Small RNA libraries were prepared using the TruSeq small RNA library preparation kit (Illumina). The resulting cDNA was amplified by PCR and all small RNA sequences were purified on gels and sequenced on a NextSeq500 (Illumina). The libraries from two stroke patient samples were of low quality and were therefore excluded from further analysis. Reads were trimmed for adapters and reads shorter than 15 nucleotides in length were discarded. Small RNAs were aligned to the human genome (hg19) and miRbase (v20) using sRNAbench [29]. miRNA counts were normalized in R Bioconductor package edgeR [30] using Trimmed Mean of M-values (TMM). Fold changes were based on the average count in each patient group.

Exiqon RNA extraction protocol and miRNA qPCR assays

RNA extractions and sample preparations were done by Exiqon Services, Denmark. Total RNA was extracted from 200 µl CSF using spin column chromatography (miRCURY™ RNA isolation kit for bio fluids). RNA was reverse transcribed using the miRCURY LNA™ Universal RT miRNA kit (Exiqon). Each miRNA was assayed once by qPCR on the miRNA Ready-to-Use PCR, Human panel I containing 372 specific miRNA primers. Amplification was performed in a LightCycler® 480 Real-Time PCR System (Roche) in 384 well plates. Using NormFinder [31] the best normalizer was identified as the average Ct value of the 9 miRNAs detected in all samples (miR-15a-5p, miR-21-5p, miR-23a-3p, miR-23b-3p, miR-99a-5p, miR-125b-5p, miR-145-5p, miR-204-5p, and miR-320a) and relative expression levels (RELs) were calculated by the formula $REL = 2^{miR\ average - miR_j}$ $2^{Global\ mean\ average - Global\ mean}$ based on the principle of $2^{-\Delta\Delta Ct}$ [32]. Fold changes were based on the median value of REL in each patient group (missing values excluded).

Norgen Biotek RNA extraction protocol and applied biosystems qPCR assays

Total RNA was extracted from 200 µl CSF using spin column chromatography (Total RNA Purification Kit, Norgen Biotek). Reverse transcription was performed by TaqMan microRNA Reverse Transcription Kit performed on a 2720 Thermal Cycler (Applied Biosystems). miRNA specific pre-amplification was accomplished using TaqMan PreAmp master mix and TaqMan MicroRNA Assays (Applied Biosystems). Pre-amplified samples and TaqMan miRNA assays for 24 selected miRNAs were applied to a 48.48 Dynamic Array IFC chip and amplified on a BioMark real-time microfluidic PCR system (Fluidigm).

Normfinder [31] identified two equally good normalizers: miR-320a and the average Ct value of the 20 miRNAs detected in all samples. Based on the assumption that most of the 24 miRNAs in this validation experiment were differentially expressed between our patient groups we wanted to avoid a global mean normalization that could potentially undermine any differences by comparing raw data to the average of all miRNAs. Therefore, miR-320a was chosen as normalizer and RELs were calculated using the formula $REL = 2^{miR\ average - miR_j}$ $2^{miR-320a\ average - miR-320a}$ based on the principle of $2^{-\Delta\Delta Ct}$ [32]. Fold changes were based on the median value of REL in each patient group (missing values excluded).

Statistics

Two-sided nonparametric Mann-Whitney tests were performed for REL comparisons between groups (SPSS software). In the NGS experiment differentially expressed miRNAs were detected by an exact test based on conditional maximum likelihood (CML) included in the R Bioconductor package edgeR [30]. *P* values from NGS and qPCR were corrected for multiple testing with the Benjamini-Hochberg (BH) procedure and *p* < 0.05 were considered significant. In all group comparisons missing expression values were treated as zero. The NGS heat map was generated using Multiple Experiment Viewer [33]. Selection of the best miRNA predictors was based on logistic lasso regression [34] performed on the top 15 most deregulated miRNAs detected by NGS. AUC and classification accuracy was estimated by leave-one-out cross-validation based on the seven predictors selected by the lasso. Differences in total numbers of miRNAs between groups as well as differences in age were analyzed by two-sided parametric *t* tests. Fischer's exact test was used for analysis of sex differences.

Results

Overall detection by NGS and Exiqon qPCR (human panel I)

A total of 246 miRNAs were detected in the CSF by NGS of which 71 miRNAs were expressed in more than 90% of the patient samples. Slightly fewer, 210 miRNAs,

were detected by the qPCR screening (Exiqon, human panel I) of which only 21 miRNAs were expressed in more than 90% of the samples. With the exception of miR-1246 all of the differentially expressed miRNAs between stroke patients and controls were detected by both NGS and qPCR (Additional file 4) although the detection rate was generally lower in the qPCR experiment.

Results from NGS

In the NGS experiment only two miRNAs (miR-1246 and miR-128-3p) were significantly up-regulated in the stroke group compared to controls after BH correction. The up-regulation of miRNAs was most evident in patients with large-artery atherosclerosis or cardioembolism whose infarcts were larger (mean volume (range) = 16.5 cm³ (2.5–32.4)) compared to patients with small-artery occlusion (mean volume (range) = 0.7 cm³ (0.3–1.6)) (Fig. 1). When dividing patients into groups according to infarct size 8 miRNAs (miR-9-5p, miR-99b-5p, miR-103a-3p, miR-107, miR-128-3p, miR-181a-5p, miR-181b-5p, and miR-1246) were significantly up-regulated in patients with infarcts >2.5 cm³ ($n = 7$) compared to stroke patients with smaller infarcts ($n = 12$).

Results from qPCR screening (Exiqon, human panel I)

Consistent with our previous findings [27] qPCR (human panel I, Exiqon) detected only 21 frequently expressed miRNAs in CSF of which none were differentially regulated between stroke patients and controls after BH correction. The up-regulation of miR-221-3p and let-7c-5p found in our previous pilot study could not be validated. By considering the expression in subgroups with different

infarct sizes two miRNAs (miR-9-3p and miR-124-3p) were significantly up-regulated in stroke patients with infarcts >2 cm³ ($n = 8$) compared to stroke patients with smaller infarcts ($n = 13$).

Validation of selected miRNAs by qPCR (applied biosystems)

Following the completion of NGS and the first qPCR experiment we selected miRNAs that showed differential expression between stroke patients and controls (and/or between patients with small and large infarcts) for technical validation. The selection was based on significant unadjusted p values ($p < 0.05$) and/or a large fold change ($0.3 > FC > 3$) in at least one of the two experiments. A total of 21 selected miRNAs were included in the validation study (normalizers excluded). Table 2 summarizes results for the 21 selected miRNAs measured with NGS, qPCR (Exiqon), and qPCR (Applied Biosystems), respectively. Five miRNAs (miR-9-5p, miR-9-3p, miR-107, miR-124-3p, and miR-128-3p) were consistently up-regulated in the group of patients with infarcts >2 cm³, although not statistically significant throughout all measurement platforms. At least four of them (miR-9-5p, miR-9-3p, miR-124-3p, and miR-128-3p) are known as brain-enriched miRNAs with a high tissue specificity index [35]. miRNA-1246 showed the highest up-regulation by NGS (FC = 13.6; $p = 0.01$) but did not appear to be up-regulated by qPCR (Applied Biosystems) and was unfortunately not included in the human panel I (Exiqon). Fig. 2 illustrates the expression levels of the four brain-enriched miRNAs in different patient subgroups.

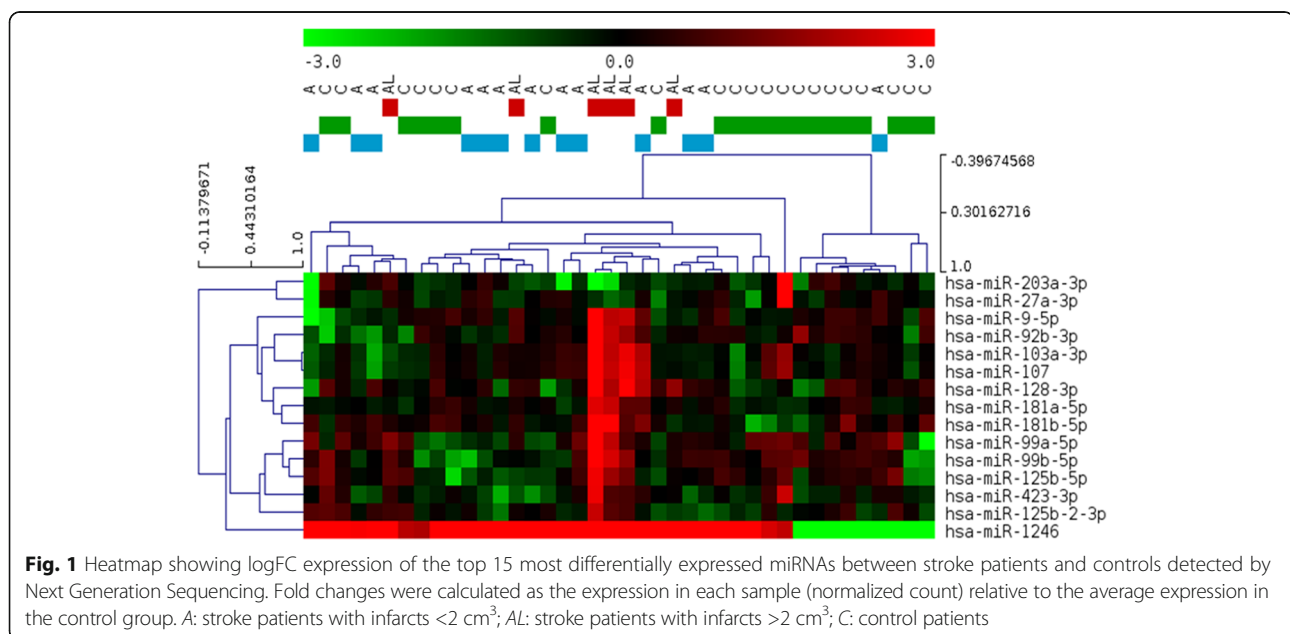
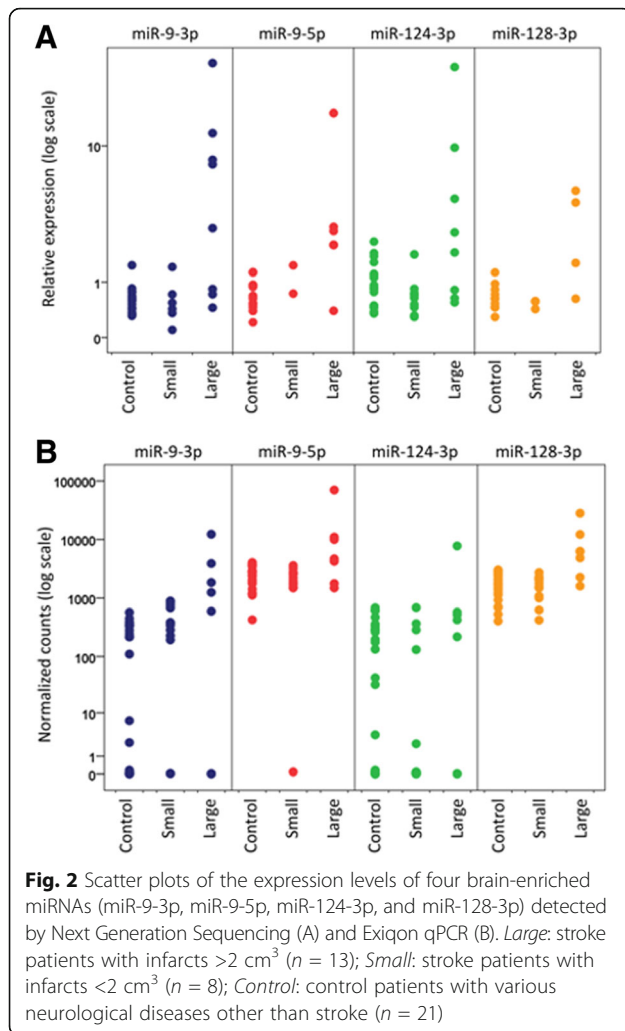


Table 2 Expression of 21 selected miRNAs detected by three different methods

miRNA	NGS				Exiqon qPCR				Applied Biosystems qPCR			
	Detection(%)	Stroke/Control FC (P)	Large/Small FC (P)	Detection(%)	Stroke/Control FC (P)	Large/Small FC (P)	Detection(%)	Stroke/Control FC (P)	Detection(%)	Stroke/Control FC (P)	Large/Small FC (P)	
let-7b-5p	95	0.8 (0.99)	2.3 (0.73)	98	1.1 (0.99)	0.6 (0.29)	100	1.0 (0.89)	100	1.0 (0.89)	0.6 (0.57)	
let-7c-5p	100	0.7 (0.99)	0.9 (0.99)	74	0.7 (0.99)	0.6 (0.11)	95	0.7 (0.71)	95	0.7 (0.71)	0.3 (0.88)	
miR-9-5p	98	2.8 (0.06)	7.0 (0.00)	48	3.3 (0.90)	2.3 (0.07)	100	0.9 (0.95)	100	0.9 (0.95)	10.0 (0.01)	
miR-9-3p	70	7.1 (0.08)	6.8 (0.28)	70	1.4 (0.85)	9.8 (0.01)	100	1.2 (0.73)	100	1.2 (0.73)	1.9 (0.66)	
miR-15a-5p	63	1.0 (0.99)	2.0 (0.99)	100	1.0 (0.50)	2.2 (0.31)	100	1.0 (0.96)	100	1.0 (0.96)	2.4 (0.44)	
miR-30d-5p	100	1.2 (0.99)	1.3 (0.79)	91	1.6 (0.45)	1.1 (0.48)	100	1.7 (0.01)	100	1.7 (0.01)	6.2 (0.09)	
miR-99b-5p	100	1.9 (0.32)	4.2 (0.00)	38	0.7 (0.99)	0.9 (0.84)	72	0.4 (0.43)	72	0.4 (0.43)	0.5 (0.70)	
miR-103a-3p	100	1.9 (0.17)	4.3 (0.00)	48	1.0 (0.84)	0.4 (0.80)	100	0.6 (0.49)	100	0.6 (0.49)	0.9 (0.85)	
miR-107	100	2.0 (0.13)	4.5 (0.00)	31	0.6 (0.71)	n.d. small	100	3.6 (0.37)	100	3.6 (0.37)	3.5 (0.58)	
miR-124-3p	58	2.7 (0.99)	11.0 (0.19)	83	0.9 (0.99)	3.6 (0.03)	100	1.1 (0.92)	100	1.1 (0.92)	1.7 (0.74)	
miR-128-3p	100	2.5 (0.04)	5.5 (0.00)	41	1.0 (0.95)	2.7 (0.15)	100	0.6 (0.42)	100	0.6 (0.42)	1.5 (0.88)	
miR-129-5p	60	2.6 (0.99)	2.3 (0.99)	26	1.1 (0.91)	3.1 (0.14)	100	1.5 (0.97)	100	1.5 (0.97)	1.0 (0.77)	
miR-181a-5p	100	1.5 (0.40)	3.0 (0.00)	33	0.9 (0.78)	0.6 (0.49)	100	0.5 (0.33)	100	0.5 (0.33)	0.7 (0.76)	
miR-181b-5p	100	1.8 (0.32)	3.1 (0.02)	5	n.d. control	n.d. small	100	0.9 (0.46)	100	0.9 (0.46)	0.7 (0.58)	
miR-203a-3p	100	0.3 (0.02)	0.8 (0.99)	-	-	-	100	0.7 (0.85)	100	0.7 (0.85)	1.4 (0.78)	
miR-204-5p	100	0.8 (0.99)	0.6 (0.73)	100	0.8 (0.90)	0.6 (0.13)	100	0.8 (0.46)	100	0.8 (0.46)	0.7 (0.06)	
miR-210-3p	38	1.3 (0.99)	2.3 (0.99)	38	1.1 (0.99)	0.4 (0.06)	100	0.9 (0.52)	100	0.9 (0.52)	1.0 (0.81)	
miR-221-3p	95	1.0 (0.99)	3.0 (0.63)	79	1.1 (0.97)	0.3 (0.33)	81	0.7 (0.32)	81	0.7 (0.32)	0.4 (0.52)	
miR-485-5p	72	2.4 (0.99)	5.5 (0.46)	-	-	-	100	0.9 (0.54)	100	0.9 (0.54)	2.2 (0.73)	
miR-497-5p	28	2.9 (0.99)	2.6 (0.99)	74	1.0 (0.94)	4.1 (0.32)	79	1.6 (0.44)	79	1.6 (0.44)	3.9 (0.65)	
miR-1246	78	11.5 (0.02)	13.6 (0.01)	-	-	-	100	1.2 (0.40)	100	1.2 (0.40)	1.0 (0.80)	

Detection (%): percentage out of 42 samples in which the miRNA was detected; Stroke/Control FC: fold change of median miRNA expression levels in the stroke group (n = 21) compared to the control group (n = 21); Large/Small FC: fold change of median miRNA expression levels in a stroke subgroup with infarcts >2 cm³ (n = 8) compared to all other stroke patients (n = 13); n.d.: not detected. All P values were adjusted for multiple testing with the Benjamini-Hochberg procedure

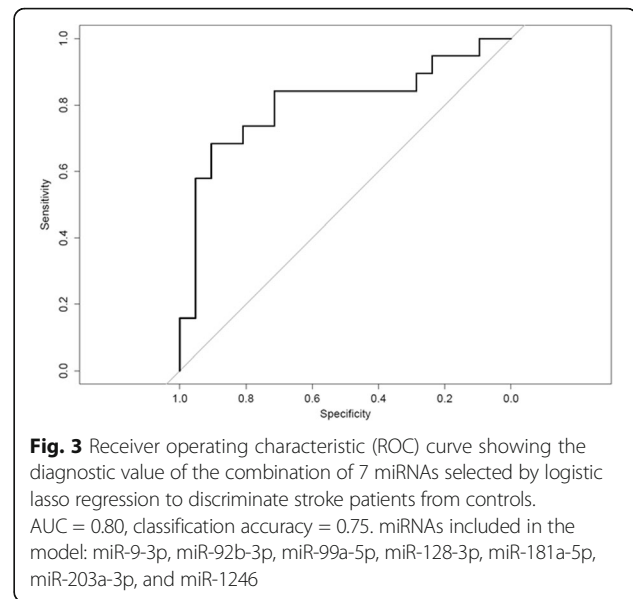


Classification accuracy and ROC curve

Although the strongest miRNA changes were seen in patients with larger infarcts we attempted to analyze the predictive power of one or more miRNAs to separate stroke patients from controls. Logistic lasso regression performed on the top 15 most differentially expressed miRNAs detected by NGS identified a combination of 7 miRNAs that could discriminate stroke patients from controls with a classification accuracy of 0.75. Fig. 3 shows the receiver operating characteristic (ROC) curve estimated by leave-one-out cross validation ($\text{AUC} = 0.80$).

Discussion

Based on the hypothesis that acute stroke causes transcriptomic changes in the brain tissue which is reflected in the molecular profile of miRNAs in the CSF, we aimed to search for a miRNA expression profile that would uniquely identify stroke patients from other neurological patients. Such a marker could potentially be useful in situations where the diagnosis may be uncertain (e.g. transient



ischemic attacks) or when the underlying cause of ischemic stroke remains unknown.

Obtaining a blood sample for measurement of miRNA biomarkers is less invasive than a lumbar puncture. However, many organs are likely to contribute to the extracellular miRNA content in plasma. Blood cells, in particular, have been shown to be major sources of plasma miRNAs. Pritchard et al. [36] showed that 58% of plasma miRNAs, reported in the literature to be candidate biomarkers for various cancers, are highly expressed in blood cells. This raises the concern that many miRNAs reported as circulating biomarkers reflect a secondary effect of blood cells rather than a disease-specific effect. Since CSF is separated from the circulation by the BBB and because of the continuous interchange between CSF and interstitial fluid [37], it seems more likely that the composition of miRNAs in CSF reflects CNS pathology better than that of blood plasma. We therefore chose to examine miRNA expression in cell-free CSF samples.

In this comprehensive investigation of miRNAs in the CSF from 21 stroke patients and 21 controls, encompassing both NGS and two different qPCR protocols, we identified relatively few frequently expressed miRNAs in the CSF. In general, the expressional variations within groups and small fold changes between groups resulted in no statistically differences for most of the detected miRNAs. However, it is noteworthy that brain-enriched miR-9-5p, miR-9-3p, miR-124-3p, and miR-128-3p were consistently up-regulated in the group of patients with infarcts $>2 \text{ cm}^3$ throughout all experiments.

Studies investigating the abundance of miRNAs in different human organs have identified miR-9-3p, miR-9-5p, miR-124-3p, and miR-128-3p as being enriched in various brain regions (midbrain, cortex, cerebellum and

hippocampus) as well as being brain-specific with a high tissue specificity index [35, 38]. Cell studies suggest that miR-124 and miR-128 are predominantly expressed in neurons [39] and studies on embryonic stem cells imply that miR-9 and miR-124 play key roles in neural fate determination in mammalian brain development [40].

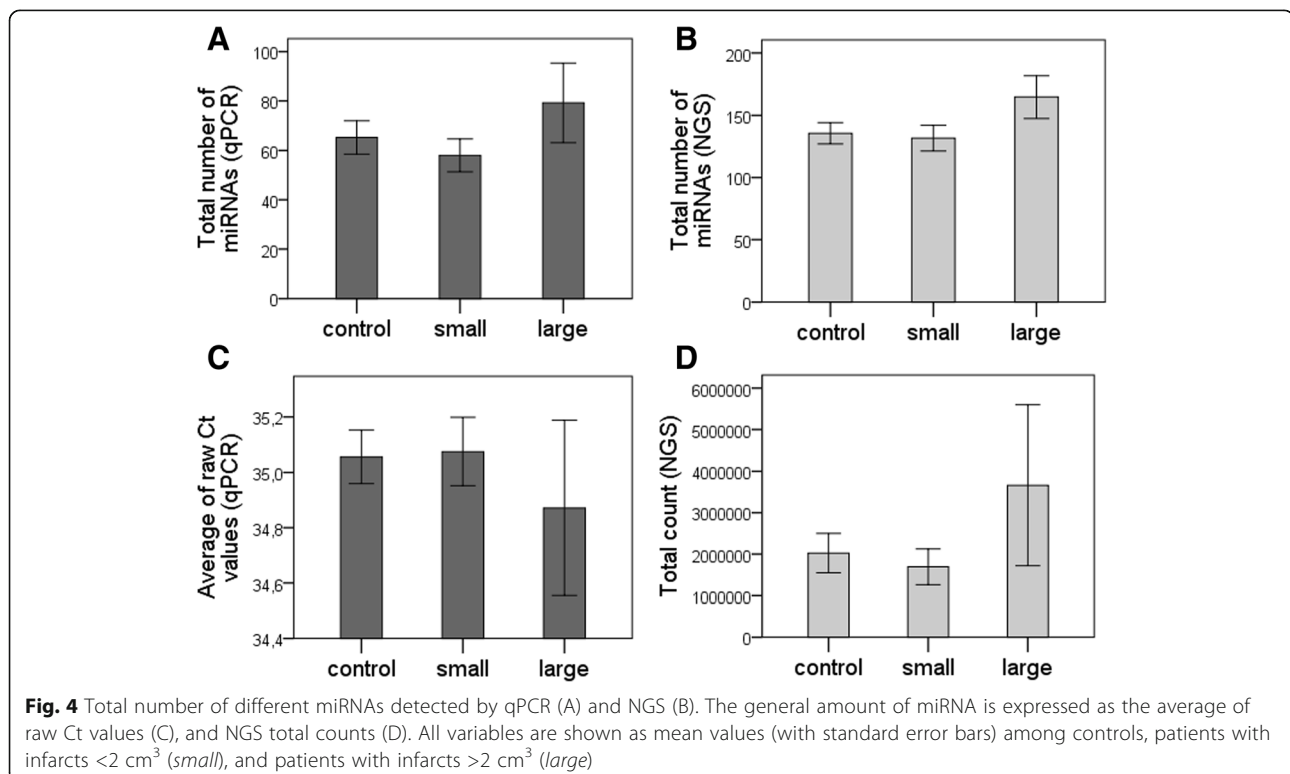
It is unknown whether disease-associated miRNAs in CSF are involved in the pathogenic mechanisms of ischemic cell death in the CNS or reflect a passive molecular response to cerebral damage. Stroke-associated miRNAs in the CSF may originate from cytoplasmic protein complexes released during neuronal and glial cell lysis caused by brain ischemia. In this study, the overall amount of miRNA in the CSF seemed slightly higher in patients with larger infarcts compared to other stroke patients, although not statistically significant. The total number of different miRNAs detected by both qPCR and NGS was significantly higher in the group of patients with infarcts $>2 \text{ cm}^3$ ($p = 0.03$), which might indicate a general leak of miRNAs into the CSF following ischemic cell injury and ensuing cell lysis (Fig. 4).

Another possible origin of any miRNA in the CSF is active release of miRNAs enclosed in small membranous vesicles (exosomes) which may play a role in intercellular communication [41, 42]. Only one study has focused on exosome-derived miRNAs in stroke patients. Ji et al. [12] extracted exosomes from serum of 65 stroke

patients and 66 non-stroke controls with matching age and cardiovascular risk factors. They found a generally higher concentration of exosomes and a significant increase of exosome derived miR-9 (FC = 16) and miR-124 (FC = 4) in the stroke group compared to controls, suggesting that exosomes from the brain can somehow cross the BBB and enter the peripheral circulation. Accordingly, Ogata et al. [43] have shown elevation of miR-9-3p in the hippocampus as well as in exosomes in peripheral blood of mice after administration of trimethyltin that selectively damages CNS neurons, indicating that miR-9-3p is a potential marker of brain damage.

If the elevation of specific miRNAs in the CSF of stroke patients is caused by expressional changes in the brain tissue, these changes could possibly both reflect the molecular chaos in the ischemic brain tissue as well as its counterregulatory mechanisms. To search for possible molecular functions we made a combined miRPath [44] (version 3.0) analysis of the up-regulated miR-9-3p, miR-9-5p, miR-107, miR-124-3p, and miR-128-3p (results merged by genes union, FDR correction, $P < 0.05$) which identified several KEGG pathways relevant to ischemic stroke, including pathways involved in p53 signaling, cell cycling, tumor necrosis factor (TNF) signaling, hypoxia inducible factor 1 (HIF-1) signaling and neurotrophin signaling.

In literature, the expression of miR-128 has been linked to alterations in the expression of genes implicated in



apoptosis (Bcl-2, Bax, p53, and Bak) as well as angiogenesis (VEGF) [45]. Yang et al. [46] found miR-128b significantly up-regulated in plasma from ischemic stroke patients compared to healthy controls. In addition, miR-124 has previously been associated with ischemic stroke. Liu et al. [47] as well as Sun et al. [48] found an increase of miR-124 in the ischemic penumbra after permanent middle cerebral artery occlusion in mice. In both studies cell culture experiments indicated that miR-124 is involved in apoptosis. Recently, Li et al. [13] reported up-regulation of three miRNAs in serum of stroke patients including miR-1246, which had the highest fold change (FC = 13.6; $p = 0.01$) in our NGS experiment. In summary, these results, although not fully comparable, support our findings. Only one study besides ours has focused on miRNAs in CSF of stroke patients. Peng et al. [49] analyzed the expression of let-7e and miR-338 in CSF from ischemic stroke patients and found up-regulation of let-7e in a group of patients included 1–7 days after symptom onset ($n = 11$) but they did not include any brain-specific miRNAs in their analysis.

Despite our consistent experimental setup, uniform sample handling, and data processing we were not able to validate the results from our previous pilot study [27]. This might be due to the small differences found in our pilot study, or the fact that none of our pilot study p values passed a BH correction for multiple testing. These results highlight the importance of performing follow-up studies, especially when patient numbers are small. Exiqon's protocol resulted in many missing values and had a lower detection rate for many miRNA sequences compared to NGS, which might explain why any potential differences in brain-enriched miRNAs were not detected in our previous pilot study. In addition, poorly elucidated factors like heterogeneity of genetic background, differences in BBB function, CSF turnover, and miRNA degradation might have influenced results differently in the two studies.

Limitations of our study include the relatively small study population and heterogeneous control group with regard to diagnoses. The various diagnoses represented in our control group may have influenced the fold changes calculated between stroke patients and controls. Nevertheless, if a stroke biomarker should be useful in the clinic, it should possess enough power to predict the diagnosis of ischemic stroke even when tested against non-healthy individuals. Therefore, and because of ethical issues in relation to doing lumbar punctures on healthy individuals, we decided to use neurological patients as controls.

We initially planned to include three equal groups of stroke patients with small-artery occlusion, large-artery atherosclerosis, and cardioembolism. However, we had to exclude patients from lumbar puncture if they were unable to give informed consent, received anticoagulant

treatment, or had large infarctions with emerging vasogenic edema which contraindicated a lumbar puncture. Therefore, our study population mainly consisted of patients with lacunar infarctions and a smaller group of patients with larger infarcts having either cardioembolism or large-artery atherosclerosis, which prevented us from comparing miRNA expression profiles in subgroups based on stroke etiologies alone.

An advantage of our study is that it takes into consideration the technical uncertainties related to miRNA profiling by using three different profiling techniques on the same sample set. We observed quite significant discrepancies between results from NGS, qPCR (Exiqon), and qPCR (Applied Biosystems). The low quantities of miRNAs in the CSF combined with multiple factors such as RNA extraction quality, affinity of the primers, and PCR efficiency could influence the results in unpredictable ways. In addition, unknown biological factors such as BBB function and CSF clearance may have influenced our results.

Although the strongest miRNA expressional changes were seen in patients with larger infarcts, we attempted to analyze the predictive power of combining one or more miRNAs to separate stroke patients from controls with logistic lasso regression based on data from NGS. The best model combined 7 miRNAs but resulted in an overall unsatisfactory classification accuracy of 0.75.

Conclusion

In conclusion, several brain-enriched miRNAs are elevated in the CSF three days after stroke onset, suggesting that these miRNAs reflect the brain damage caused by ischemia. The expressional differences seem, however, limited to patients with larger ischemic injury, which argues against the use of CSF miRNAs as diagnostic biomarkers of stroke based on current methods.

Additional files

Additional file 1: Diagnosis, stroke subtype, infarct volume, and NIHSS of each patient included. (DOCX 20 kb)

Additional file 2: Comparison between study populations in our previous and present study. (DOCX 17 kb)

Additional file 3: Full description of the different protocols used for miRNA. (DOCX 31 kb)

Additional file 4: Venn diagram of miRNAs detected by NGS and qPCR extraction and miRNA profiling. (DOCX 192 kb)

Abbreviations

AUC: Area under the curve; BBB: Blood brain barrier; BH: Benjamini-Hochberg; CML: Conditional maximum likelihood; CNS: Central nervous system; CSF: Cerebrospinal fluid; Ct value: Cycle threshold value; CT: Computed tomography; FDR: False discovery rate; HIF-1: Hypoxia inducible factor 1; IFC chip: Integrated fluidic circuit chip; KEGG: Kyoto Encyclopaedia of Genes and Genomes; miRNAs: MicroRNAs; MRI: Magnetic resonance imaging; NGS: Next Generation Sequencing; qPCR: Quantitative Polymerase Chain Reaction; REL: Relative expression value; ROC: Receiver Operating

Characteristics; TMM: Trimmed Mean of M-values; TNF: Tumor necrosis factor; TOAST: Trial of Org 10,172 in Acute Stroke Treatment; VEGF: Vascular endothelial growth factor

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SS participated in the design of the study, included the patients, collected the CSF samples, participated in the laboratory analyses and the data management, and drafted the manuscript. AN participated in the design of the study, helped to plan the laboratory analyses, contributed to the data management, and reviewed the manuscript. AC planned and carried out the qPCR (Applied Biosystems), contributed to the data management, and reviewed the manuscript. NH helped to organize the laboratory analyses, contributed to the data management, and reviewed the manuscript. MB participated in the design of the study, planned and carried out the NGS, contributed to the management and interpretation of data, and reviewed the manuscript. TC conceived the study, helped to plan and to organize the study, helped to include the patients, contributed to the interpretation of data, and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All project procedures were approved by the Danish Research Ethics Committee (project ID: H-1-2011-094) and were in accordance with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients prior to inclusion. Patients did not receive any fee for participation in the study.

Consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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References

- Hart RG, Diener HC, Coutris SB, Easton JD, Granger CB, O'Donnell MJ, et al. Embolic strokes of undetermined source: the case for a new clinical construct. *Lancet Neurol*. 2014;13:429–38.

- Kernan WN, Ovbiagele B, Black HR, Bravata DM, Chimowitz MI, Ezekowitz MD, et al. AHA / ASA guideline guidelines for the prevention of stroke in patients with stroke and transient ischemic attack; 2014. doi:10.1161/STR.0000000000000024.
- Diener H, Eikelboom J, Connolly SJ, Joyner CD, Hart RG, Lip GYH, et al. Apixaban versus aspirin in patients with atrial fibrillation and previous stroke or transient ischaemic attack: a predefined subgroup analysis from AVERROES, a randomised trial. *Lancet Neurol*. 2017;11:225–31.
- Hart RG, Benavente O, McBride R, Pearce LA. Antithrombotic therapy to prevent stroke in patients with atrial fibrillation: a meta-analysis. *Ann Intern Med*. 1999;131:492–501.
- Randomised trial of endarterectomy for recently symptomatic carotid stenosis: final results of the MRC European Carotid Surgery Trial (ECST). *Lancet*. 1998;351:1379–87.
- Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of org 10172 in acute stroke treatment. *Stroke*. 1993;24:35–41.
- Goldstein LB, Jones MR, Matchar DB, Edwards LJ, Hoff J, Chaturvedi V, et al. Improving the reliability of stroke subgroup classification using the trial of ORG 10172 in acute stroke treatment (TOAST) criteria. *Stroke*. 2001;32:1091–7.
- Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010;56:1733–41.
- Glinge C, Clauss S, Boddum K, Jabbari R, Jabbari J, Risgaard B, et al. Stability of circulating blood-based MicroRNAs – pre-analytical methodological considerations. *PLoS One*. 2017; doi:10.1371/journal.pone.0167969.
- Balzano F, Deiana M, Giudici SD, Oggiano A, Baralla A, Pasella S, et al. MiRNA stability in frozen plasma samples. *Molecules*. 2015;20:19030–40.
- Bam M, Yang X, Sen S, Zumbun EE, Dennis L, Zhang J, et al. Characterization of Dysregulated miRNA in peripheral blood mononuclear cells from ischemic stroke patients. *Mol Neurobiol*. 2017; doi:10.1007/s12035-016-0347-8.
- Ji Q, Ji Y, Peng J, Zhou X, Chen X, Zhao H, et al. Increased brain-specific MiR-9 and MiR-124 in the serum Exosomes of acute ischemic stroke patients. *PLoS One*. 2016;11:e0163645.
- Li P, Teng F, Gao F, Zhang M, Wu J, Zhang C. Identification of circulating MicroRNAs as potential biomarkers for detecting acute ischemic stroke. *Cell Mol Neurobiol*. 2015;35:433–47.
- Wang W, Sun G, Zhang L, Shi L, Zeng Y. Circulating MicroRNAs as novel potential biomarkers for early diagnosis of acute stroke in humans. *J Stroke Cerebrovasc Dis*. 2014;23:2607–13.
- Tsai P-C, Liao Y-C, Wang Y-S, Lin H-F, Lin R-T, Juo S-HH. Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease. *J Vasc Res*. 2013;50:346–54.
- Hossmann K-A. Pathophysiology and therapy of experimental stroke. *Cell Mol Neurobiol*. 2006;26:1057–83.
- Christensen T. Experimental Focal Cerebral Ischemia - pathophysiology, metabolism and pharmacology of the ischemic penumbra. Copenhagen: University of Copenhagen; 2007. p. 1–56. http://curis.ku.dk/ws/files/107825836/Disputats_TChristensen.pdf.
- Schmidt-Kastner R, Zhang B, Belayev L, Khoutorova L, Amin R, Busto R, et al. DNA microarray analysis of cortical gene expression during early recirculation after focal brain ischemia in rat. *Brain Res Mol Brain Res*. 2002;108:81–93.
- Lu XCM, Williams AJ, Yao C, Berti R, Hartings JA, Whipple R, et al. Microarray analysis of acute and delayed gene expression profile in rats after focal ischemic brain injury and reperfusion. *J Neurosci Res*. 2004;77:843–57.
- Kinouchi H, Sharp FR, Hill MP, Koistinaho J, Sagar SM, Chan PH. Induction of 70-kDa heat shock protein and hsp70 mRNA following transient focal cerebral ischemia in the rat. *J Cereb Blood Flow Metab*. 1993;13:105–15.
- Dharap A, Bowen K, Place R, Li L. Transient focal ischemia induces extensive temporal changes in rat cerebral MicroRNAome. *J Cereb Blood Flow Metab*. 2009;29:675–87.
- Liu D-Z, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, et al. Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *J Cereb Blood Flow Metab*. 2010;30:92–101.
- Wang W-X, Huang Q, Hu Y, Stromberg AJ, Nelson PT. Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. *Acta Neuropathol*. 2011;121:193–205.
- Wang C, Pan Y, Cheng B, Chen J, Bai B. Identification of conserved and novel microRNAs in cerebral ischemia-reperfusion injury of rat using deep sequencing. *J Mol Neurosci*. 2014;54:671–83.

25. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke*. 2008;39:959–66.
26. Gubern C, Camós S, Ballesteros I, Rodríguez R, Romera VG, Cañadas R, et al. miRNA expression is modulated over time after focal ischaemia: up-regulation of miR-347 promotes neuronal apoptosis. *FEBS J*. 2013;280:6233–46.
27. Sørensen SS, Nygaard A-B, Nielsen M-Y, Jensen K, Christensen T. miRNA expression profiles in cerebrospinal fluid and blood of patients with acute ischemic stroke. *Transl Stroke Res*. 2014;5:711–8.
28. Garcia-Finana M, Cruz-Orive LM, Mackay CE, Pakkenberg B, Roberts N. Comparison of MR imaging against physical sectioning to estimate the volume of human cerebral compartments. *NeuroImage*. 2003;18:505–16.
29. Rueda A, Barturen G, Lebron R, Gomez-Martin C, Alganza A, Oliver JL, et al. SRNAtoolbox: An integrated collection of small RNA research tools. *Nucleic Acids Research*. 2015. doi:10.1093/nar/gkv555.
30. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2009;26:139–40.
31. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR Data : a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64:5245–50.
32. Jolla L. Quantification strategies in real-time PCR. In: Bustin S, editor. *A-Z of Quantitative PCR*. La Jolla: International University Line (IUL); 2004. p. 87–112.
33. Saeed A, Sharov V, White J. TM4: a free, open-source system for microarray data management and analysis. *BioTechniques*. 2003;34:374–7.
34. Tibshirani R. Regression selection and shrinkage via the lasso. *J R Stat Soc B*. 1996;58:267–88.
35. Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, et al. Distribution of miRNA expression across human tissues. *Nucleic Acids Res*. 2016;44:3865–77.
36. Pritchard CC, Kroh E, Wood B, Arroyo JD, Dougherty KJ, Miyaji MM, et al. Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev Res (Phila)*. 2012;5:492–7.
37. Jessen NA, Munk ASF, Lundgaard1 I, Nedergaard M. The Glymphatic system – a Beginner’s guide. *Neurochem Res*. 2016;8:583–92.
38. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*. 2007;129:1401–14.
39. Zeng Y. Regulation of the mammalian nervous system by MicroRNAs. *Mol Pharmacol*. 2009;75:259–64.
40. Krichevsky AM, Sonntag K-C, Isacson O, Kosik KS. Specific microRNAs modulate embryonic stem cell-derived neurogenesis. *Stem Cells*. 2006;24:857–64.
41. Lee Y, El Andaloussi S, Wood MJ a. Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. *Hum Mol Genet*. 2012;21:R125–34.
42. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9:654–9.
43. Ogata K, Sumida K, Miyata K, Kushida M, Kuwamura M, Yamate J. Circulating miR-9* and miR-384-5p as potential indicators for trimethyltin-induced neurotoxicity. *Toxicol Pathol*. 2015;43:198–208.
44. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, et al. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res*. 2015; doi:10.1093/nar/gkv403.
45. Adlakha YK, Saini N. Brain microRNAs and insights into biological functions and therapeutic potential of brain enriched miRNA-128. *Mol Cancer*. 2014;13:1–18.
46. Yang Z-B, Li T-B, Zhang Z, Ren K-D, Zheng Z-F, Peng J, et al. The diagnostic value of circulating brain-specific MicroRNAs for ischemic stroke. *Intern Med*. 2016;55:1279–86.
47. Liu X, Li F, Zhao S, Luo Y, Kang J, Zhao H, et al. MicroRNA-124-mediated regulation of inhibitory member of apoptosis-stimulating protein of p53 family in experimental stroke. *Stroke*. 2013;44:1973–80.
48. Sun Y, Gui H, Li Q, Luo Z-M, Zheng M-J, Duan J-L, et al. MicroRNA-124 protects neurons against apoptosis in cerebral ischemic stroke. *CNS Neurosci Ther*. 2013;19:813–9.
49. Peng G, Yuan Y, Wu S, He F, Hu Y, Luo B. MicroRNA let-7e is a potential circulating biomarker of acute stage ischemic stroke. *Transl Stroke Res*. 2015;6:437–45.

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