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The Role of miRNA Expression Profiles in Different Biofluids İn Aneurysm Rupture

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Abstract

Objective : Intracranial aneurysm (IA) is a cerebrovascular disease in which the cerebral arteries become pathologically weakened. The molecular mechanisms behind the pathogenesis of IAs are poorly understood. MicroRNAs are highly stable in body fluids and the expression signatures of specific circulating miRNAs may be associated with high rupture risk, severity, and clinical outcome of SAH.

Methods : The presented study aimed to detect miRNA-based biomarkers and evaluating the usability of blood for a non-invasive approach. Blood samples from 24 patients with unruptured IA (Group 1), blood and CSF samples collected on day five after aSAH from 24 patients with ruptured IA (Group 2), and both the blood and CSF samples from 24 individuals without any positive IA history (Control group) were subjected to qRT-PCR for evaluating the expression profiles of 8 miRNAs.

Results : MiR-29a, miR-200a-3p, miR-451a, miR-1297, and miR-502-5p in blood and miR-29a, miR-200a-3p, miR-451a, miR-126, miR-146a-5p, and miR-27b-3p in CSF were found to be differentially expressed in ruptured patients compared to controls. In both biofluids of ruptured cases, the differences in the expression profiles of miR-29a, miR-200a-3p, and miR-451a compared to controls were striking. The upregulation of miR-126, miR-200a-3p, miR-451a, and miR-502-5p in the ruptured group compared to unruptured patients suggesting that these miRNAs may be informative in predicting the risk of an aneurysmal rupture.

Conclusion : MiR-29a, miR-200a-3p, and miR-451 were significantly altered in patients with aSAH compared to controls in both biofluids. These findings suggest that these miRNAs could be candidate non-invasive biomarkers for aSAH.

Key Words : Intracranial aneurysm · Subaracnoid hemorrhage · miRNA · Biomarker.

INTRODUCTION

The common cerebrovascular disease, intracranial aneurysm (IA), is a local pathological enlargement of the cerebral arteries caused by the destruction of the inner elastic plate and becoming weak to withstand hemodynamic pressure (2). IA is present in 2-5% of the general population worldwide and usually remains asymptomatic (14,26). However, rupture of the aneurysm and subsequent massive intracranial and subarachnoid hemorrhage (SAH) are associated with 70% mortality and 30–50% morbidity in the cases (7). Aneurysmal SAH (aSAH) accounts for only 5% of all strokes, but it has a major impact due to its destructive effects (5). Various risk factors have been identified for aneurysmal growth and rupture. These factors can be patient-specific factors such as age, female gender, hypertension, smoking, alcohol use, geographic region, and family history, or they can be aneurysm-specific factors, such as aneurysm location, irregular aneurysm morphology and the size of aneurysm (9,10). Therefore, identifying the cases at high risk of ruptured aneurysms in the early stage would be necessary to prevent severe sequelae. Up to now, the diagnosis of IAs has been based on imaging techniques. However, the management of aneurysms, the decision to intervene, and selecting an adequate clinical method are challenging for the physicians, and these approaches do not allow for determining the possibility of rupture (26,36). Studies have shown that microRNAs are keys to most protein processing and can be identified in biological fluids as potential early biomarkers for various cerebrovascular diseases and play roles in IAs (38). Since it is not possible to reach the risk of possible rupture IA through the imaging techniques used today, by analyzing circulating miRNAs in biological fluids, it may be possible to reach noninvasive approaches that can help in the diagnosis of early rupture and aSAH and prognostic evaluation. MicroRNAs (miRNAs) are small (18-22 nucleotides in length) nonprotein coding RNAs that play a critical role in post-transcriptional regulation of gene expression by interfering with transcription or inhibiting translation. MiRNAs regulate almost all biological processes, including cell development, differentiation, proliferation, apoptosis, etc, and they are degredation resistant and highly stable in body fluids such as extracellular serum, plasma, and cerebrospinal fluid (CSF) (14,18). Previous studies have shown that deviations from normal miRNA functions often lead to impaired

cellular and biological functions, which may contribute to the development and progression of related diseases. In connection with these effects, circulating miRNAs have been shown to have the potential for diagnostic and prognostic biomarkers in many diseases, including cancer, cardiovascular, immunological, and neurodegenerative diseases (15).

However, specific and sensitive biomarkers that can predict the formation, progression and rupture of IAs have not been detected so far. Studies have reported that changes in the profile of certain circulating miRNAs may be associated with high rupture risk, severity and clinical outcome of SAH and can also be detected in the early stages of disease development (14).

The presented study aimed to detect miRNA-based biomarkers that can be highly reliable in differential diagnosis by comparing the miRNA expression profiles of the cases with and without rupture with a non-invasive approach. In order to achieve the aim of determining the usability of the non-invasive approach, differences in the expression profiles of selected miRNAs in both peripheral blood and CSF samples of IA cases with rupture were evaluated. To the best of our knowledge, studies evaluating miRNA expression profiles in both samples of cases are very limited.

MATERIALS AND METHODS

Ethics statement

This study was conducted according to the guidelines presented in the Declaration of Helsinki for research experiments involving human subjects, and approved by the Institutional Ethics Review Board of the XX University, Medical Faculty (2023-48). Each individual provided written informed consent.

Literature research

Preliminary research was conducted to select miRNAs reported to have the potential to reveal the risk of aSAH among IAs by examining relevant articles and databases such as Pubmed, Embase, Ovid, Google Scholar, and miRbase. As a result of these studies, the expression profiles of 8 miRNAs (miR-29a, miR-200a-3p, miR-451a, miR-126, miR-146a-5p, miR-1297, miR-502-5p, and miR-27b-3p) (1,6,35,40,42,16,21,23,25,31–34) in the peripheral blood and CSF samples of the IA cases were determined and compared with the control group CSF and blood samples.

Patient recruitment

Study participants were recruited from the Neurology and Neurosurgery departments of the XX University Hospital, Turkey. The diagnosis of both IAs and aSAH was based on the clinical examination and neuro-imagings including computed tomography (CT), computed tomography angiography (CTA), digital subtraction angiography (DSA) and magnetic resonance angiography (MRA) as defined in the World Health Organization (WHO) criteria. The cases with a positive past medical history for cardiovascular, immunological diseases, cancer, or organ failure that may have the potential to change miRNA expression levels were not included in the study. Clinically healthy volunteers without a positive family history of IA/aSAH and/or neurological and chronic/systemic diseases were included to the study as controls. Not only baseline demographic data but also details regarding vascular risk factors, such as hypertension, diabetes, smoking, and alcohol consumption, were gathered from all participants.

Study design

The study was conducted in three groups, and all samples obtained from individuals who met the previously explained inclusion criteria were analyzed in this study,

Group 1: Peripheral blood samples from 24 patients diagnosed with unruptured IA (UIA) (24 samples).

Group 2: Peripheral blood and CSF samples from 24 patients ruptured IA (RIA) (48 samples). The samples were collected on day 5 after SAH.

Group 3: Peripheral blood and CSF samples from 24 individuals without positive disease history (48 samples). CSF samples were collected from them in which lumbar puncture was performed as part of subarachnoid anaesthesia treatment before lower extremity orthopaedic surgery. They gave their written informed consent for participation in the study.

Whole blood and CSF samples collection

The peripheral blood samples of all patients and controls were collected into PAXgene Blood RNA tubes (PreAnalytiX, GmbH, Switzerland). To ensure complete lysis of blood cells, the tubes were incubated at room temperature for at least 2h according to the modified manufacturer's protocol and then fractioned into multiple aliquots and stored at −80 °C until further analysis.

The CSF samples taken from RIA and controls via lumbar drainage were centrifuged for 5 minutes at 2000 g at +4°C to destroy contaminated blood cells. CSF material was taken from the tube with a pipette without disturbing the blood pellet, and then aliquots were stored at −80 °C until RNA isolation and further analysis.

RNA extraction from whole blood and CSF samples

Total RNAs from peripheral blood and CSF samples were extracted by using PAXgene Blood miRNA kit (PreAnalytiX), and miRVANA™ PARIS™ Kit (Invitrogen by Thermo Fisher Scientific), respectively, according to the instructions of the manufacturers. RNA concentration was quantitated by the NanoDrop ND-1000 Spectrophotometer (PeQLab Biotechnologie, GmbH), and the ratio of OD260 and OD280 absorbance of each sample was in between 1.9–2.1.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Complementary DNA (cDNA) was synthesized using the miRNA ALL-In-One cDNA Synthesis Kit (ABM, CA) following the manufacturer's directives. Each reaction mixture utilized 2X cDNA was created using the miRNA ALL-In-One cDNA Synthesis Kit (ABM, CA) according to the manufacturer's instructions. Each reaction mixture contained 2X miRNA cDNA Synthesis SuperMix (10 µl), 2 µl Enzyme Mix, 2 µl total RNA, and 6 µL nuclease-free water, resulting in a total reaction volume of 20 µl. The reverse transcription process followed the cycle profile of 37°C for 30 min, 50°C for 15 min, 85°C for 5 min, and a hold at 4°C.

Quantitative real-time PCR reaction (qRT-PCR) was carried out on a CFX-96 Real-Time PCR Detection System (BIO-RAD, C1000 Touch Thermal Cycler) using BrightGreen miRNA qPCR Master Mix (5 µl), forward primer (0.35 µl), reverse primer (0.35 µl), cDNA (2 µl), and nuclease-free water (2.3 µl) to make the final volume 10 µl (ABM, CA). The amplification reaction conditions were: incubation at 95 ˚C for 10 minutes, followed by 40 cycles of 95 ˚C for 10 seconds, 60 ˚C for 20 seconds, and 72 ˚C for 30 seconds. The PCR reaction was performed in duplicates.

The primer catalogue numbers for miR-126, miR-29a, miR-200a-3p, miR-451a, miR-146a-5p, miR-1297, miR-27b-3p, and miR-502-5p were MPH01082, MPH01310, MPH02296, MPH02756, MPH02204, MPH01130, MPH02385, and MPH01742, respectively (ABM, CA). The primer sequences are given in Table 1.

The cycle threshold (Ct) values of the reference and target miRNA were determined in each sample, and the relative miRNA level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The statistical analysis was conducted using SPSS version 21.0 (IBM Corporation, NY, USA), while GraphPad Prism v.9.5.1 (Graph Pad Software) was used to generate the graphs. The Shapiro-Wilk test was employed to evaluate the distribution of the variables. For normal variation variables, comparisons were made using the Student t test, while the Mann–Whitney U test was used to determine differences between the two groups. The diagnostic potential of candidate miRNAs as a biomarker was assessed by constructing Receiver operating characteristic (ROC) curves for miRNAs with a significant difference in levels and calculating the area under ROC curve (AUC), specificity, and sensitivity with a 95% confidence interval (95% CI). A value of $P<0.05$ was considered statistically significant.

RESULTS

General and clinical characteristics of study participants

Seventy-two study subjects, including 24 UIA patients, 24 RIA patients, and 24 healthy controls, were enrolled in this study. Blood samples were collected from UIA patients, whereas both the blood and CSF samples were taken from the RIA patients on day 5 after the onset of SAH. Among 48 IA cases, 22 were male and 26 were female (mean age 53.04±13.02 years; range 29-79). Healthy controls comprised 10 males and 14 females (mean age 61.42±11.42). The general characteristics of controls and IA patients are summarized in Table 2.

Differentially expressed miRNAs in IA patients versus healthy controls in whole blood and CSF

While no significant difference was detected between miRNA expression levels in the blood samples of UIA patients and healthy individuals, it was observed that the miRNA expression profiles of patients with RIA changed significantly compared to controls. MiR-29a (FC=6.22, p=0.033), miR-200a-3p (FC=19.66,

p=0.002), miR-451a (FC=8.93, *p*=0.014), miR-1297 (FC=11.24, *p*=0.025), and miR-502-5p (FC=10.60, *p*=0.044) were significantly upregulated in the RIA group compared with controls (Figure 1A). To evaluate the diagnostic accuracy of these circulating miRNAs, we performed ROC curve analysis for each miRNA individually. The five upregulated miRNAs had a significantly high AUC: 0.836, 0.929, 0.857, 0.821, and 0.771 for miR-29a, miR-200a-3p, miR-451a, miR-1297 and miR-502-5p, respectively (Figure 1B). These findings suggest that these miRNAs can discriminate ruptured aneurysms patients from healthy controls. Table 3 shows AUC, specificity, sensitivity, and cut-off values based on the ROC curve analysis.

We have detected six miRNAs in CSF samples that show a difference in levels between the patients with RIA and controls. Expression levels of miR-126, miR-200a-3p, miR-451a, and miR-146a-5p were significantly higher in RIA patients compared to controls, with fold changes of 47.23, 9.75, 7.45, and 9.45, respectively. On the other hand, miR-29a and miR-27b-3p levels were significantly lower in RIA cases compared to controls, with fold changes of 3.74 and 2.65, respectively. Please refer to Table 4 and Figure 2A for more information. Our results revealed that four upregulated miRNAs (miR-126, miR-200a-3p, miR-451a, and miR-146a-5p) had an AUC of 0.889, 0.889, 0.900, and 0.891, respectively. In contrast, the two downregulated miRNAs had a significantly high AUC:0.950 and 0.848 for miR-29a and miR-27b-5p, respectively (Table 5, Figure 2B). MiR-29a, miR-200a-3p, and miR-451 achieved AUCs greater than 0.8 in two biofluids, demonstrating that these miRNAs had a high discriminating power to distinguish ruptured aneurysm patients from healthy controls. These findings suggest that these three miRNAs may serve as potential non-invasive biomarkers for aSAH.

Differentially expressed miRNAs in ruptured aneurysm versus upruptured aneurysm in whole blood

When the miRNA expression profiles between the RIA and UIA patients were compared, it was observed that the expression levels of four miRNAs (miR-126 (FC=9.43, *p*=0.001), miR-200a-3p (FC=11.47,

p=0.005), miR-451a (FC=5.84, *p*=0.043), and miR-502-5p (FC=10.66, *p*=0.044)) were significantly upregulated in the RIA patients compared to unruptured patients (Figure 3A). The AUC values for distinguishing RIA and UIA cases were 0.897, 0.855, 0.770, and 0.729 for miR-126, miR-200a-3p, miR-451a, and miR-502-5p, respectively (Table 6, Figure 3B). These miRNAs were found to be increased only in ruptured patients, not in unruptured patients, suggesting that these miRNAs may be informative in predicting the risk of an aneurysmal rupture.

DISCUSSION

MicroRNAs are small molecules involved in various biological fluids, including serum, plasma, and cerebrospinal fluid. They regulate various biological processes, including apoptosis, neuroinflammation, oxidative stress, cerebral edema, neurogenesis, and angiogenesis. They also play a significant role in maintaining vascular integrity and function and are highly sensitive to cellular stimuli and pathophysiological conditions. Therefore, changes in miRNA expression in body fluids can directly reflect ongoing acute pathophysiological events (37,41).

Despite significant advances in treatment, it remains a significant cause of morbidity and mortality due to the lack of biomarkers that would allow for the screening and timely intervention of high-risk aneurysms. Therefore, there is an urgent need to identify robust markers with high sensitivity and specificity for accurate identification of aSAH or prediction of aneurysmal rupture.

Firstly, Jin et al. (17) evaluated different serum miRNA expressions between healthy individuals and aneurysm patients, as well as between different stages of the aneurysm. They reported that miRNAs may play a role in the regulation of the formation and development of intracranial aneurysms and may also have a warning effect for ruptured IAs. Likewise, Li et al. (20) also reported that circulating miRNAs can be used as new biomarkers in identifying high-risk IAs. Since then, the search for informative miRNAs in

plasma and/or CSF samples of RIA and UIA cases has continued with different molecular methods, but their diagnostic and prognostic performance still needs improvement.

In this study, the levels of specific miRNAs were analyzed in blood and CSF samples of the UIA, RIA patients, and healthy controls. Our results showed a statistically significant increase in the expressions of miR-29a, miR-200a-3p, miR-451a, miR-1297, and miR-502-5p in the blood of aSAH patients compared with healthy controls. Additionally, when miRNA expressions were compared in the CSF samples of RIA patients with the control group samples, different expression profiles of 6 miRNAs were detected. Interestingly, miR-29a, whose high expression was observed in the blood sample, was significantly downregulated in CSF samples compared to controls. In ROC analyses performed to evaluate the diagnostic performance of CSF and blood miRNAs in diagnosing aSAH, AUC values of selected miRNAs revealed that they could discriminate between aSAH patients and controls.

Moreover, the expressions of miR-29a, miR-200a-3p, and miR-451a in both biofluid samples of aSAH patients were significantly changed with high predicted probability (AUC > 0.8) compared to controls. These findings suggest that these miRNAs may serve as potential non-invasive biomarkers for aSAH. In addition, we observed significantly different expressions of miR-126, miR-200a-3p, miR-451a, and miR-502-5p between the RIA and UIA groups (AUC: 0.897, 0.855, 0.770, and 0.729, respectively). The levels of these miRNAs were increased only in ruptured cases, suggesting that they influence the risk of aneurysmal rupture.

The miR-29 family are widely expressed in vascular tissues and have been suggested to play an important role in maintaining the integrity of arteries (4). MiR-29a has been reported to play a role in the fibrotic response mediated by collagen I, collagen III, fibrin-1, and elastin-1 and in the regulation of gene transcripts encoding extracellular matrix proteins (3). Du et al. suggested a close relationship between decreased miR-29a expression and an increased risk of excessive collagen deposition in proximal tubule cells, thus suggesting that miR-29a may participate in IA progression by adversely regulating the expression of collagen, which is responsible for vessel wall elasticity (8). Wang and colleagues reported that miR-29a

expression was higher in peripheral blood samples of aneurysm cases than in control individuals and that there was a significant relationship between aneurysm rupture rate and miR-29a expression (40). The in vitro study by Zhao et al. also revealed that miR-29a downregulation reduced the apoptosis of vascular smooth muscle cells in the human brain, while miR-29a overexpression increased the apoptosis in these smooth muscle cells. They also confirmed using in vivo IA models that miR-29a overexpression could induce apoptosis through mitochondrial pathways and concluded that miR-29a may contribute to IA progression by regulating mitochondrial apoptotic pathways (44).

Interestingly, in our study, it was found that miR-29a expression in CSF samples of ruptured cases, unlike in blood samples, was significantly reduced compared to controls, and in the ROC analysis, the AUC reached 0.95 with 100% specificity. Since miR-29a expression is directly related to the regulation of collagen expression, which is responsible for vessel wall elasticity, and to the apoptosis of vascular smooth muscle cells, the down-regulation of miR-29a with a high AUC predictive value seems to be a candidate biomarker in evaluating the risk of ruptured aneurysms.

Experimental studies have shown that aSAH can induce morphological and functional changes and apoptosis in the vascular endothelium, causing "endothelial dysfunction." Due to endothelial dysfunction, ECs cannot perform basal functions effectively cannot produce sufficient NO, leading to vascular narrowing, and coagulation and permeability functions cannot be controlled (11,28). Moreover, apoptotic damage to the endothelium is critical because it compromises blood–brain barrier integrity, disrupts physiological vasoregulation, and increases smooth muscle cell proliferation and blood coagulation. Recently, it has been reported that the miR-200 family is closely related to the functions of vascular endothelial cells and blood vessels. The overexpression of it was associated with increased EC permeability (19,39). It is a type of angiogenesis signal regulatory factor important in maintaining the integrity of vascular endothelial cells and blood vessels in the body.

In our study, it was observed that miR-200a expression increased significantly in blood and CSF fluid samples of the cases with ruptured aneurysm compared to controls. These data suggest that the miR-200a-

3p expression profile is directly related to the risk of aneurysmal rupture, as also stated in previous studies (14,25,30), and may have a potential for being an informative biomarker in identifying individuals at high risk of IA development and subsequent rupture.

In our study, miR-451a expression increased in IA cases compared to controls, and this increase was significantly higher in aSAH cases than in non-ruptured cases. Previous studies have reported that miR-451a expression levels, commonly used in panels to assess hemolysis in aSAH cases, differ from controls. Lopes et al. reported that miR-451a expression in the whole blood of patients with and without vasospasm was significantly downregulated 7–10 days after aSAH, and its association with clinical outcomes may also be related to the rupture or destruction of red blood cells (23).

MiR-126 is one of the most abundant miRNAs in endothelial cells and is known to play a vital role in regulating endothelial cell function, maintaining vascular integrity, accelerating post-ischemic angiogenesis, as well as endovascular inflammation and platelet activation (27,43). Salajegheh et al. (29) reported that miR-126 directly targets vascular endothelial growth factor (VEGF) to regulate tumor proliferation. VEGF is an important vascular growth factor. Knockout of miR-126 in mice and zebrafish reduced vascular integrity and impaired the proliferation, migration, and angiogenesis activity of endothelial cells (12,13). In the study of Liu et al., inflammatory smooth muscle cells have been reported to affect the progression of IA by regulating VEGF expression and inducing endothelial cell changes (22).

Similar to our study, Iwuchukwu et al. have compared the miRNA expression profiles of peripheral blood and CSF samples collected within 24 h of presentation from the patients with intracerebral hemorrhage, and they reported downregulated expression of miR-126 in CSF samples compared to the plasma in the patients. They have suggested that decreased expression of miRNA-126 may lead to the expression of VCAM-1, and they hypothesized that miRNA profile in CSF is physiologically close to profiles in brain extracellular fluid. Hence, the miRNA content differences between these biofluids may reflect an altered systemic inflammatory response leading to neuroinflammation and blood-brain barrier damage (16).

In our study, among the miRNA expressions that increased significantly in CSF samples of rupture IA patients compared to control samples, the highest increase (47-fold) was miR-126 expression. Moreover, when the peripheral blood samples of IA with and without rupture were compared, miR-126 expression was significantly increased in rupture cases, consistent with the previous studies (24,42). Recent studies have reported that aSAH cases can be used as a biomarker to predict aneurysmal ruptures due to significantly increased circulating miR-126 expression levels compared to controls and non-bleeding IAs; our findings also support this view.

Despite these discussions, our study has some limitations. The small sample size was an important limitation in terms of increasing the diagnostic and prognostic accuracy of relevant miRNAs. In addition, only one sampling time (day five after SAH) was considered negative in terms of revealing the expression changes of miRs in blood and CSF. Because it has been demonstrated in many studies that miRNA expression levels in biological fluids change and are dynamic following SAH. Therefore, in the larger cohort, assessing miRNA expression at various time points will help identify prognostic biomarkers and prevent complications. Despite these limitations, we believe that our study, which analyzes miRNA expressions in both blood and CSF samples of aSAH cases, is superior to related studies in the literature.

CONCLUSION

We demonstrated that miR-29a, miR-200a-3p, and miR-451 were significantly altered in patients with aSAH compared to controls in both biofluids (AUC > 0.8). Our results suggest that these miRNAs could be candidate non-invasive biomarkers for aSAH. We identified four circulating miRNAs (miR-126, miR-200a-3p, miR-451a, and miR-502-5p) that can discriminate ruptured aneurysms from unruptured ones. Future studies are needed to investigate the exact roles of these miRNAs in the progression and rupture of IAs in large cohort sizes before introduction into clinical applications.

AUTHORS' DECLARATION

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Informed consent

Informed consent was obtained from all individual participants included in this study.

Data sharing

None

Preprint

None

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Table 1. Primer sequences for qRT-PCR

miRNA	Forward 5'-3'	Reverse 5'-3'
hsa-miRNA-126-3p	UCGUAC CGUGAGUAAUAAUGCG	CAUUAU UACUUUUGGUACGCG
hsa-miR-29a-3p	UAGCACCAUCUGAAAUCGGUUA	ACCGAUUUCAGAUGGUGCUAUU
hsa-miR-200a-3p	UAACACUGUCUGGUAACGAUGU	AUCGUUACCAGACAGUGUUAUU
hsa-miR-451a	AAACCGUUACCAUUACUGAGUU	CUCAGUAAUGGUAACGGUUUUU
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU	AACCCAUGGAAUUCAGUUCUCA
hsa-miR-1297	UUCAAGUAAUUCAGGUG	CCUGAAUUACUUGAAUU
hsa-miR-27b-3p	UUCACAGUGGCUAAGUUCUGC	UAGCAGCACGUAAAUAUUGGC
hsa-miR-502-5p	AUCCUUGCUAUCUGGGUGCUA	GCACCCAGAUAGCAAGGAUUU

Table 2. General characteristics of the IA patients and controls

Parameter	Group 1 : unruptured IA blood (n=24)	Group 2 : ruptured IA blood +CSF (n=24)	Controls (blood + CSF) (n=24)
Age (mean±SD)	50.33±15.61	55.75±9.73	61.42±11.42
Sex, Male	10	12	10
Sex, Female	14	12	14
Aneurysm location			
Anterior communicating artery	4	12	
Internal carotid artery	14	8	
Anterior cerebral artery	2	-	
Middle cerebral artery	4	2	
Posterior communicating artery	-	2	

Table 3. Receiver Operating Characteristic (ROC) curve analysis for microRNAs with biomarker potential in blood samples

miRNA	Sensitivity (%)	Specificity (%)	AUC (95% CI)	Cut-off fold	*p*-value
miR-29a	90.00	71.43	0.836 (0.580-0.967)	≤3.61	0.002
miR-200a-3p	90.00	85.71	0.929 (0.696-0.997)	≤3.66	0.000
miR-451a	90.00	85.71	0.857 (0.605-0.976)	≤2.94	0.000
miR-1297	80.00	71.4	0.821 (0.563-0.961)	≤5.37	0.002
miR-502-5p	80.00	85.71	0.771 (0.508-0.936)	≤2.34	0.034

p value <0.05 is statistically significant. AUC : area under the ROC curve, CI : confidence interval

Table 4. MiRNAs with a difference in levels between ruptured IA and controls in CSF samples

miRNA	Fold change (FC)	*p*-value	Ruptured IA vs. control
miR-29a	3.74	0.002	Down-regulated
miR-200a-3p	9.75	0.019	Up-regulated
miR-451a	7.45	0.024	Up-regulated
miR-126	47.23	0.019	Up-regulated
miR-146a-5p	9.45	0.013	Up-regulated
miR-27b-3p	2.65	0.020	Down-regulated

p value <0.05 is statistically significant

Table 5. Receiver operating characteristic curve analysis for microRNAs with biomarker potential in CSF samples

miRNA	Sensitivity (%)	Specificity (%)	AUC (95% CI)	Cut-off fold	*p*-value
miR-29a	90.00	100.00	0.950 (0.691-1.000)	>5.99	0.000
miR-200a-3p	77.78	100.00	0.889 (0.610-0.991)	≤9.71	0.000
miR-451a	80.00	100.00	0.900 (0.624-0.994)	≤10.11	0.000
miR-126	88.89	100.00	0.889 (0.610-0.991)	≤8.11	0.000
miR-146a-5p	90.91	80.0	0.891 (0.636-0.989)	≤9.78	0.000
miR-27b-3p	72.73	100.00	0.848 (0.595-0.973	>8.76	0.000

p value <0.05 is statistically significant, AUC= area under the ROC curve, CI= confidence interval

Table 6. Receiver operating characteristic curve analysis for microRNAs with biomarker potential in rupture of aneurysm

miRNA	Sensitivity (%)	Specificity (%)	AUC (95% CI)	Cut-off fold	*p*-value
miR-126	100.00	81.82	0.897 (0.692-0.985)	≤4.15	0.000
miR-200a-3p	90.00	81.82	0.855 (0.633-0.968)	≤3.66	0.000
miR-451a	80.00	70.00	0.770 (0.530-0.926)	≤2.74	0.016
miR-502-5p	70.00	75.00	0.729 (0.500-0.894)	≤2.2	0.047

p value <0.05 is statistically significant, AUC= area under the ROC curve, CI= confidence interval

Table 7. Summary of the studies investigating miRNAs as candidate biomarkers in diagnosis and prognosis of aSAH

Type of sample	Study papulation	Day of collection	Method	Differential miRNAs	Main findings	Limitations	Reference
Plasma	Discovery: aSAH cases with 11 additional UIA (n=15), control (n=15) Validation: 15 aSAH, 15 UIA,15 control	2 years	PCR array, RT-PCR	Discovery cohort aSAH vs control: miR-200a-3p (FC=1.6); miR-183-5p (FC=-1.6); miR-2355-3p (FC=1.3) aSAH with additional UIA vs control: miR-141-3p (FC=1.7); miR-let-7b-5p (FC=-1.4) Validation cohort IA vs control: miR-183-5p (FC=-2.2) aSAH vs control: miR-200a-3p (FC=1.8, AUC=0.74); miR-183-5p (FC=-2.1, AUC=0.8) UIA vs control: miR-183-5p (FC=-2.4, AUC=0.83); Let-7b-5p (FC=-1.7, AUC=0.92)	miR-200a-3p may influences the risk of aneurysmal rupture. miR-183-5p and let-7b-5p had a good to excellent discriminating power for distinguishing IA patients from controls.	1. Limited sample size 2. Subgroup had additional UIA, and not of patients with UIA alone	[28]

Blood	27 aSAH (14 DCV+, 13 DCV-)	Day 7-10	NGS	aSAH vs control: let-7f-5p (FC=-2.4); miR-126-5p (FC=-4.6); miR-146a-5p (FC=2.1); miR-17-5p (FC=-1.4); miR-451a (FC=-2.2); miR-486-5p (FC=-1.8); miR-589-5p (FC=1.7); miR-941 (FC=1.5) DCV+ vs DCV-: no significant difference was detected	The association of miR-451a with aSAH clinical outcomes may be related to the rupture or destruction of red blood cells. MYC gene identified as an important target in the pathogenesis of SAH.	-	[26]
Serum	60 aSAH, 10 control	Day 3	microarray, RT-PCR	aSAH vs control: miR-502-5p (AUC=0.958); miR-1297 (AUC=0.950); miR-4320 (AUC=0.843) aSAH severity: miR-502-5p ↑and miR-1297↑= WFNS 4-5, mRS 4-6	miR-502-5p and miR-1297 levels were associated with SAH severity and poor utcome and could	1. Limited sample size 2. Samplying time point 3. Samples collected during drug therapy	[22]

					serve as candidate biomarkers in diagnosis and prognosis of SAH.		
Plasma	Discovery (20 aSAH, 20 control) Validation (68 aSAH, 90 control)	12 h post SAH	RT-PCR	Discovery cohort 76 miRNAs were differentially expressed Validation cohort aSAH vs control: miR-15a-5p (AUC=0.727); miR-34a-5p (AUC=0.763); miR-374a-5p (AUC=0.625); miR-146a-5p (AUC=0.917); miR-376c-3p (AUC=0.812); miR-18b-5p (AUC=0.930); miR-24-3p (AUC=0.883); miR-27b-3p (AUC=0.857) aSAH severity and outcome: miR-146a-5p; miR-27b-3p	miR-146a-5p and miR-27b-3p could potentially serve as a measure of aSAH severity and outcomes.	1. Limited sample size 2. Study patients with spontaneous SAH without IA	[39]
CSF	19 aSAH (10 DCV+, 9	Day 1-18	NanoString nCounter	DCV+ vs DCV-: miR-27a-3p; miR-516a-5p; miR-566; miR-1197	miR-451a may have an essential	Limited sample size	[37]

	DCV-), 4 control			aSAH vs control: 36 miRNAs exhibited fold change with significant difference. miR-451a showed significantly increase fold changes in a number of group comparisions.	regulatory role in the pathology of SAH.		
CSF, plasma	31 aSAH (13 DCV+, 18 DCV-), 8 control	Day 3,7	TaqMan Low-Density Array	DCV+ vs DCV- CSF: miR-7b-5p (AUC=1); miR-29a-3p (AUC=1); miR-17-5p (AUC=1); miR-19b-3p (AUC=1); miR-20a-5p (AUC=1); miR-24-3p (AUC=1); and 31 others plasma: miR146a-5p (AUC=0.838); miR-7a-5p (AUC=0.873); miR-204-5p (AUC=0.917); miR-221-3p (AUC=0.875); miR-23a-3p (AUC=0.90); miR-497 (AUC=0.975); and 23 others aSAH vs control	Several specific miRNAs had a high discriminating power for distinguish DCV in two biofluids.	1. Limited sample size 2. Sampling time point 3. Validate using a different method 4. Correlated the ekspression of miRNAs with another aSAH complications	[47]

				CSF: miR146a-5p, miR-29a-3p, and 26 others plasma: miR146a-5p, miR-27b-3p, and 16 others			
Whole blood	Acute phase: 19 aSAH; chronic phase: 20 aSAH; and 20 control	Acute: first 72 h Chronic: 3-15 months	NGS	Acute, chronic and control: 106 mature miRNA and 90 miRNA precursors were differentially expressed (miR-7f-5p, miR-451a, miR-148a, and 100 others)	The top pathway of altered miRNAs are related to inflammation and the immune response, especially those related to cytokine receptor interactions	1. Limited sample size 2.Validation	[20]
CSF, blood	24 UIA, 24 aSAH, 24 control	Day 5	RT-PCR	aSAH vs control CSF: miR-29a (FC=3.74, AUC=0.950); miR-200a-3p (FC=9.75, AUC=0.889); miR-451a (FC=7.45, AUC=0.900); miR-126	miR-29a, miR-200a-3p, and miR-451 could be candidate biomarkers for aSAH. miR-	1. Limited sample size 2. Samplying time point 3. Validate in cell and	Our study

				(FC=47.23, AUC=0.889); miR-146a-5p (FC=9.45, AUC=0.891); miR-27b-3p (FC=2.65, AUC=0.848) Blood: miR-29a (FC=6.22, AUC=71.43); miR-200a-3p (FC=19.66, AUC=0.929); miR-451a (FC=8.93, AUC=0.857); miR-1297 (FC=11.24, AUC=0.821); miR-502-5p (FC=10.60, AUC=0.771) aSAH vs UIA (blood): miR-126 (FC=9.43, AUC=0.897); miR-200a-3p (FC=11.47, AUC=0.855); miR-451a (FC=5.84, AUC=0.770); miR-502-5p (FC=10.66, AUC=0.729)	126, miR-200a-3p, miR-451a, and miR-502-5p may influences the risk of aneurysmal rupture.	animal models 4. Resolution of the method we used	

IA: intracranial aneurysm; aSAH: aneurysmal subarachnoid hemorrhage; UIA: unruptured IA; RIA: ruptured IA; DCV: delayed cerebral vasospasm; FC: Fold Change; AUC: area under the ROC curve

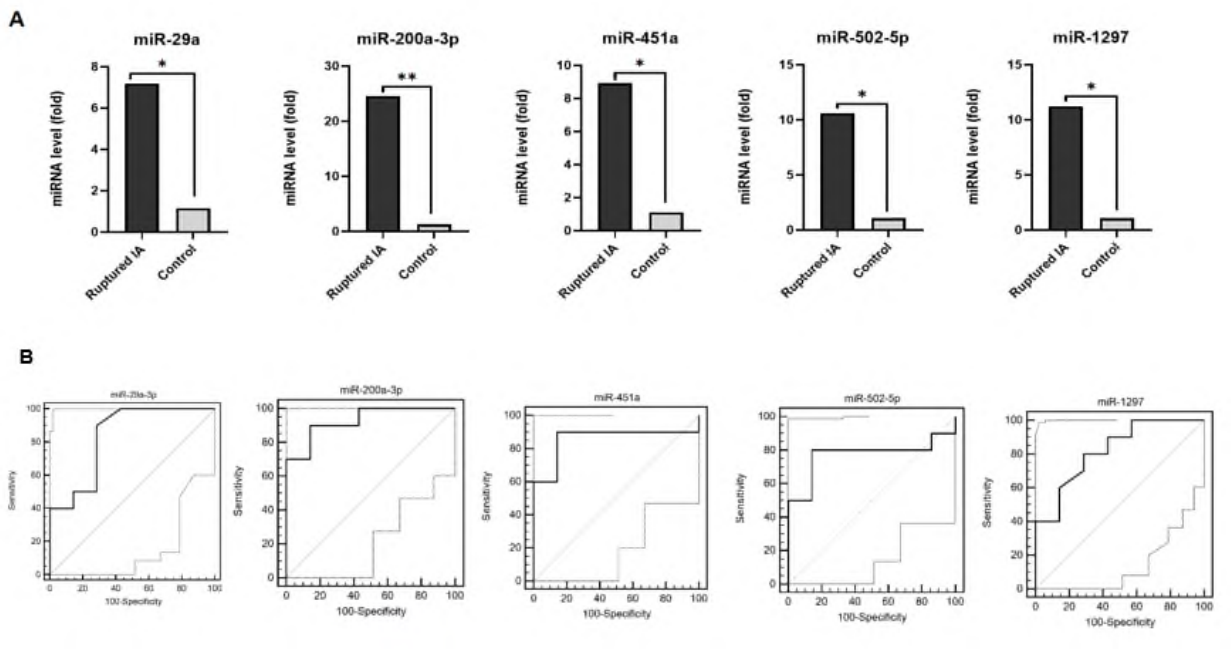

Fig. 1. The differential expression levels of five blood miRNAs in the patients with ruptured aneurysm and healthy controls. (A) Relative expression of miRNAs in ruptured aneurysm patients and controls is presented in bar graphs. All miRNA levels were presented as fold changes. Data are shown as mean. (B) Receiver operating characteristic (ROC) curve analysis of the five upregulated miRNAs. The AUC of miR-29a, miR-200a-3p, miR-451a, miR-1297, and miR-502-5p was 0.836, 0.929, 0.857, 0.821 and 0.771, respectively. Significant differences are marked with asterisks as follows: ***p*=0.001-0.01, **p*=0.01-0.05. AUC, area under the curve

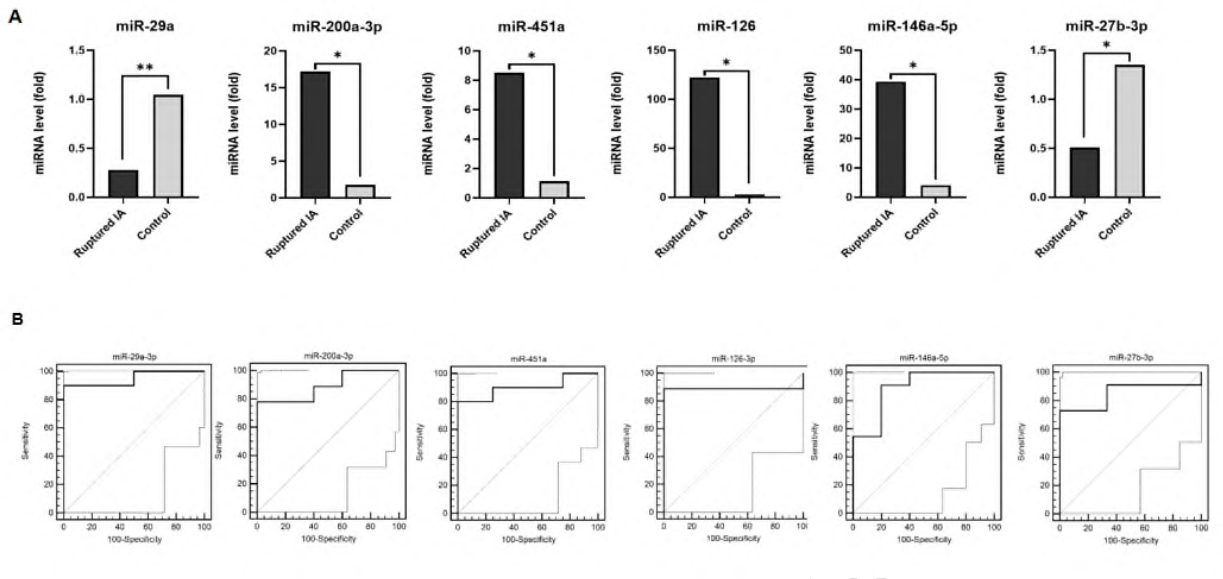

Fig. 2. The differential expression level of six CSF miRNAs (four upregulated and two downregulated) in the patients with ruptured aneurysm and healthy controls. (A) Relative expression of miRNAs in ruptured aneurysm patients and controls is presented in bar graphs. All miRNA levels were presented as fold changes. Data are shown as mean. (B) The ROC curve analysis of upregulated and downregulated miRNAs. The AUC of miR-29a, miR-200a-3p, miR-451a, miR-126, miR-146a-5p, and miR-27b-3p was 0.950, 0.889, 0.900, 0.889, 0.891, and 0.848, respectively. Significant differences are marked with asterisks as follows: ***p*=0.001-0.01, **p*=0.01-0.05. AUC, area under the curve

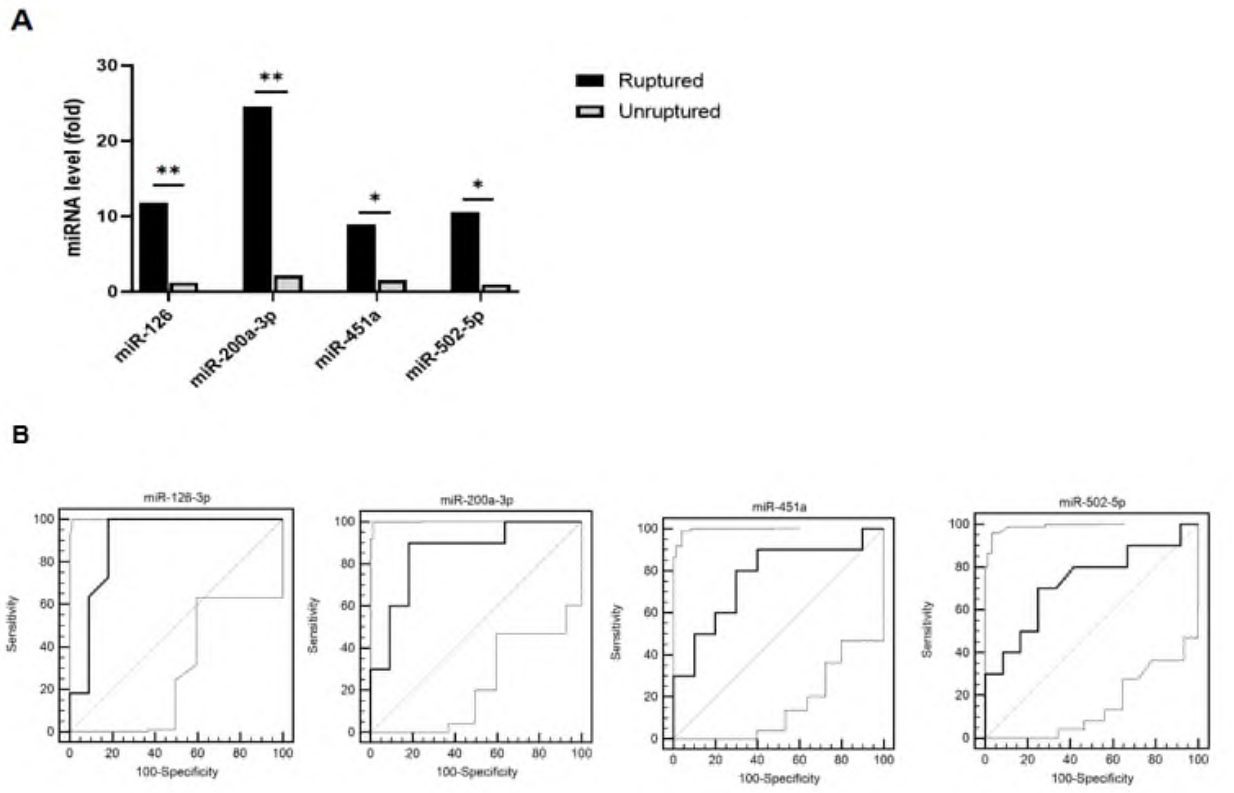

Fig. 3. Differentially expressed miRNAs in patients with ruptured aneurysm and unruptured aneurysm. (A) Relative levels of four miRNAs with a difference in levels between RIA and UIA cases. All miRNA levels were presented as fold changes. Data are shown as mean. (B) Receiver operating characteristic (ROC) curve analysis of the four upregulated miRNAs. The AUC of miR-126, miR-200a-3p, miR-451a and miR-502-5p was 0.897, 0.855, 0.770 and 0.729, respectively. Significant differences are marked with asterisks as follows: ***p*=0.001-0.01, **p*=0.01-0.05. AUC, area under the curve.