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Evaluation of the Role of miRNAs Expression Profiles in Aneurysm

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ABSTRACT

AIM: To evaluate the diagnostic and prognostic significance of miRNA signatures by identifying differences in miRNA expression between ruptured and unruptured intracranial aneurysm (IA) cases, as well as to pinpoint miRNAs that correlate with clinical severity in patients with aneurysmal subarachnoid hemorrhage (aSAH).

MATERIAL and METHODS: Peripheral blood samples were collected from 50 IA patients (25 without rupture and 25 with rupture) and 25 healthy controls. In the ruptured group, analyses were performed on samples collected on Days 3 and 5 after SAH. The clinical severity and outcomes of the disease were assessed using Fisher grades, WFNS grades, Hunt–Hess grades, and the Modified Rankin Scale.

RESULTS: We found that the expression levels of miR-21-5p and miR-15a were significantly altered in unruptured aneurysms (UA) patients compared to controls. The expression levels of 10 miRNAs were significantly decreased in ruptured aneurysms (RA) patients compared to controls. The ruptured group also exhibited an upregulation of 16 miRNAs relative to the unruptured group. Furthermore, we noted a significant increase in miR-24 expression in RA patients between Days 3 and 5, suggesting its potential role in the progression of aSAH. miR-9p-3p and miR-497 were found to be associated with aSAH severity. Moreover, the levels of miR-451a, miR-146a-5p, miR-502-5p, and miR-497 were significantly lower in patients with poor outcomes compared to those with favorable outcomes.

CONCLUSION: These findings suggest that specific miRNAs may serve as potential diagnostic and prognostic biomarkers for IA and subsequent SAH, particularly on Day 3 following aSAH.

KEYWORDS: Intracranial aneurysm, Aneurysmal subarachnoid hemorrhage, miRNA, Biomarker, Preipheral blood

ABBREVIATIONS: aSAH: Aneurysmal subarachnoid hemorrhage, **AUC:** Area under the ROC curve, **CSF:** Cerebrospinal fluid, **CVS:** Cerebral vasospasm, **DCI:** Delayed cerebral ischemia, **DCV:** Delayed cerebral vasospasm, **FC:** Fold change, **IA:** Intracranial aneurysm, **miRNAs:** MicroRNAs, **mRS:** Modified rankin scale, **RA:** Ruptured aneurysms, **ROC:** Receiver operating characteristic, **SAH:** Subarachnoid hemorrhage, **UA:** Unruptured aneurysms, **VSMC:** Vascular smooth muscle cells, **WFNS:** World federation of neurosurgical societies

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INTRODUCTION

Subarachnoid hemorrhage (SAH) is the extravasation of blood within a vessel into the subarachnoid space, which is normally filled with cerebrospinal fluid (CSF) in the central nervous system (27). The most common cause of non-traumatic SAH is intracranial aneurysm (IA) rupture (37). Aneurysmal subarachnoid hemorrhage (aSAH) is a severe health problem due to both the direct effects of bleeding and complications such as vasospasm (34). aSAH has an incidence of approximately 6-16 per 100,000 individuals annually worldwide, with a mortality rate of 45% (17). Various modifiable and non-modifiable factors that affect the risk of aneurysm formation, growth, and rupture, such as smoking, alcohol use, female gender, and a family history of IA, have been identified (40). Although there have been remarkable advances in the diagnosis, treatment, and follow-up of the disease, it remains a significant cause of morbidity and mortality due to a 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 the accurate identification of aSAH or prediction of aneurysmal rupture (38).

MicroRNAs (miRNAs) are single-stranded small conserved noncoding RNAs composed of 18-22 nucleotides that play critical roles in regulating gene expression at the post-transcriptional level (9). More than 2,500 miRNAs in humans have been identified as being able to regulate over 60% of protein-coding genes. These molecules play a role in various physiological processes, such as cell proliferation, cell differentiation, apoptosis, oxidative stress, and angiogenesis (46). Differential expression of miRNAs can often impair cellular and biological functions and contribute to the development and progression of disease. miRNAs are known to be involved in numerous diseases, including cancer, cardiovascular disease, and neurological conditions. Although limited, data showing that specific miRNAs may play a vital role in the development of IA and the progression of aSAH are available (9,18,26,37).

This case-control study aimed to determine miRNA expression differences in ruptured and unruptured IA cases, evaluate the diagnostic and prognostic significance of these miRNA signatures, and identify markers associated with clinical severity in aSAH patients.

MATERIAL and METHODS

Ethics Statement

This study was conducted in accordance with the guidelines presented in the Declaration of Helsinki for research experiments involving human subjects and approved by the Institutional Ethics Review Board of the Eskisehir Osmangazi University, Medical Faculty (2023-48). All participants provided written informed consent.

Literature Search

A preliminary search was conducted to identify miRNAs reported to have the potential to reveal the risk of aSAH among IA cases by examining relevant articles and databases such as PubMed, Embase, Ovid, Google Scholar, and miRbase. Based on previous studies, the expression profiles of 20 miRNAs, including miR-29a, miR-126, miR-200a-3p, miR-451a, miR-146a-5p, miR-1297, miR-27b-3p, miR-502-5p, miR-143-3p, miR-145-5p, miR-17-5p, miR-221-3p, miR-21-5p, miR-15a, miR-9-3p, miR-630, miR-24, miR-148b-3p, miR-497, and miR-183-5p (2,5,7,11,20,21,23,32-36,41,42,45,48,50-52,55), in the peripheral blood samples of IA cases were determined and compared with control blood samples.

Participants

Patients with ruptured aneurysms (RA) and unruptured aneurysms (UA) were recruited from the Neurosurgery and Neurology departments of the Medical Faculty, Eskisehir Osmangazi University, Turkey. The diagnosis of both IA and aSAH was based on clinical examination and neuro-imaging techniques, including computed tomography (CT), computed tomography angiography, digital subtraction angiography, and magnetic resonance angiography, as defined in the World Health Organization criteria. Cases with a medical history of cardiovascular or immunological diseases, cancer, and organ failure were excluded, as these conditions have the potential to change miRNA expression levels. Clinically healthy individuals of both sexes, without neurological and chronic/systemic diseases, IA, or a family history of IA/aSAH, constituted the control group of the study. Baseline demographic data, as well as details regarding vascular risk factors, such as hypertension, diabetes, smoking, and alcohol consumption, were gathered from all participants.

The present study included a total of 100 blood samples from IA/aSAH patients and controls: (A) 25 patients with unruptured IA (Group 1); (B) 25 patients with ruptured IA; samples from aSAH patients were collected post-SAH on Days 3 and 5 (Group 2); and (C) 25 healthy controls. All participants were from the Turkish population.

Whole Blood Collection and Total RNA Extraction

Venous whole blood was collected using PAXgene Blood RNA tubes (PreAnalytiX GmbH, Switzerland). To ensure the complete lysis of blood cells, each PAXgene Blood RNA tube was incubated at room temperature (15-25°) for a minimum of 2 hours before processing, per the manufacturer's protocol. Total RNA extraction was performed using the PAXgene Blood miRNA kit (PreAnalytiX, Qiagen, Switzerland) according to the manufacturer's recommendations. The RNA concentration was assessed using a NanoDrop ND-1000 spectrophotometer (PeQLab Biotechnologie GmbH), and the samples were subsequently stored at –80°C for further analysis.

Quantitative Real-Time PCR (qRT-PCR)

A total of 20 miRNAs were screened for. Reverse transcription of miRNA was carried out using the miRNA All-In-One cDNA Synthesis Kit (Cat#G898 ABM, CA, USA) following the manufacturer's instructions. Each reaction mixture consisted of 10 µl of 2X miRNA cDNA Synthesis SuperMix, 2 µl of Enzyme Mix, 2 µl of Total RNA, and 6 µL of nuclease-free water, bringing the total volume to 20 µL. Then, the reaction followed this cycling profile: 37 °C for 30 min, 50 °C for 15 min,

Table I: Clinical Parameters of IA Groups and Control Participants

Parameters	Controls (n=25)	Group 1: Unruptured IA (n=25)	Group 2: Ruptured IA (n=25)	p-value
Age (year)	61.42 ±11.28	56.32±14.06	59.12±12.20	0.585
Gender (females)	14 (56)	15 (60)	14 (56)	0.959
Hypertension	None	9 (36)	7 (28)	0.544

Data are presented as mean ± standard deviation or n (%); p value <0.05 is considered statistically significant.

85 °C for 5 min, and hold at 4 °C. Amplification was performed on a CFX-96 Real-Time PCR Detection System (C1000 Touch Thermal Cycler, BIO-RAD) with a reaction mixture containing 5 µl BrightGreen miRNA qPCR Master Mix, 0.35 µl forward primer, 0.35 µl reverse primer, 2 µl cDNA, and 2.3 µl nuclease-free water, reaching a final volume of 10 µl. U6 was used as a housekeeping gene (all from ABM, CA, USA). The RT-PCR protocol was as follows: incubation at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 10 seconds, 60 °C for 20 seconds, and 72 °C for 30 seconds. Cycle threshold (Ct) values were determined for each sample, and the relative miRNA level was calculated using the formula 2−ΔΔCt.

Statistical Analysis

Statistical analyses were performed using SPSS version 25.0 (IBM, NY, USA). The distribution of the variables was evaluated with the Shapiro–Wilk test. Comparisons were performed using Student's t-test for normally distributed variables. The Mann–Whitney U test was used to evaluate the differences between two groups, and the Kruskal–Wallis test assessed differences among more than two groups. Correlations between variables were evaluated using Spearman's rank correlation coefficient analysis. Receiver operating characteristic (ROC) curves were generated for each miRNA, and the area under the ROC curve (AUC), specificity, and sensitivity were calculated with a 95% confidence interval (95% CI) to evaluate the potential of these candidate miRNAs as diagnostic biomarkers.

RESULTS

Demographic and Clinical Features of the Study Groups

Among the 50 IA patients included in this study, 29 were female and 21 were male (mean age 57.72±13.10 years; range 29-79), and 16 had a history of hypertension. The healthy controls comprised 14 women and 11 men (mean age 61.43±11.28 years). No significant differences in age or gender were observed between the IA cases and the control cohort. The distribution of hypertension was similar in both RA and UA patients. The clinical features of the controls and IA patients are summarized in Table I. Clinical severity assessments at admission indicated that five cases (20%) were categorized as WFNS (World Federation of Neurosurgical Societies) Grades 3-5, while nine cases (36%) were classified as Hunt–Hess Grades 3-5. Brain CT results showed Fisher Grade 4 in 60% of patients. Regarding clinical outcomes, 40% of the patients showed a modified Rankin Scale (mRS) score of 3-6. The clinical characteristics of aSAH patients are presented in Table II.

Table II: Clinical Characteristics of the Patients (n=25) with aSAH

Clinical profiles	n (%)
Symptoms	
Headache	16 (64)
Vomiting	6 (24)
Altered sensorium	6 (24)
Focal deficits	2 (8)
Seizures	7 (28)
Aneurysm location	
Anterior communicating artery	13 (52)
Middle cerebral artery	4 (16)
Internal carotid artery	2 (8)
Anterior cerebral artery	1 (4)
Posterior communicating artery	1 (4)
Multiple	4 (16)
Fisher grade	
1-3	10 (40)
4	15 (60)
Hunt-Hess grade	
1-2	16 (64)
3-5	9 (36)
WFNS grade	
1-2	20 (80)
3-5	5 (20)
Modified Rankin Scale (mRS)	
1-2	15 (60)
3-6	10 (40)

WFNS: *World Federation of Neurologic Surgeons.*

Comparison of the miRNA Expression Profiles in the Blood Samples of UA Cases and the Control Group

Among the 20 examined miRNAs, two miRNAs, namely miR-21-5p and miR-15a, were differentially represented in controls and UA patients: the expression level of miR-21-5p was significantly increased (Fold Change (FC)=2.07, p=0.006), whereas miR-15a was considerably decreased (FC=0.72, p=0.049) in UA cases compared with controls (Figure 1A). The sensitivity and specificity of these circulating miRNAs as diagnostic indicators were determined by analyzing the AUC for each miRNA. miR-21-5p and miR-15a had AUC values of 0.829 (p<0.0001) and 0.780 (p=0.001), respectively (Figure 1B). The findings suggest that these two miRNAs together can discriminate UA patients from healthy individuals.

Differently Expressed miRNAs Between the Ruptured and Control Groups

Expression analyses were conducted on blood samples taken on the third and fifth days post-hemorrhage to identify miRNAs that may contribute to prognostic assessment and clinical recovery in cases of rupture. The expression levels of circulating miRNAs in blood samples taken on the third and fifth days after bleeding from ruptured cases differed from those in the control group. Specifically, nine miRNAs on the

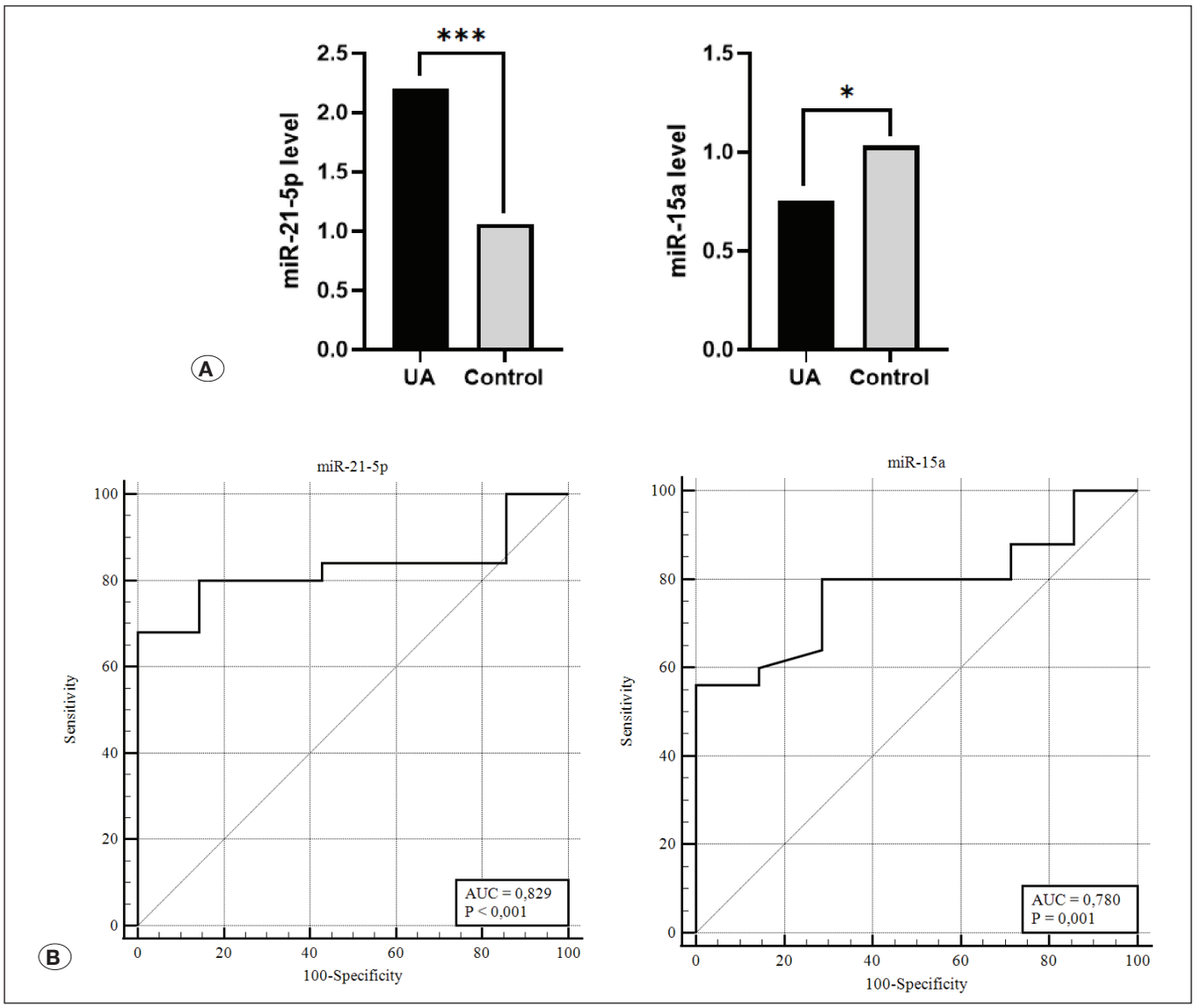


Figure 1: Expression levels of differentially expressed miRNAs in patients with unruptured aneurysms and healthy controls. **A)** Relative expression of miR-21-5p and miR-15a in unruptured aneurysm patients and controls. All miRNA levels are presented as fold changes. Data are shown as means. **B)** ROC curves to distinguish unruptured IA patients from healthy controls. The AUCs of miR-21-5p and miR-15a were 0.829 and 0.780, respectively. Statistically significant differences are marked with asterisks: ***p=0.0001-0.001, **p=0.001-0.01, *p=0.01-0.05. **AUC:** Area under the curve, **ROC:** Receiver operating characteristic.

third day after bleeding and six miRNAs on the fifth day post-bleeding were found to have statistically significant decreases in expression levels compared to the control group (Figure 2A, 2B). Subsequently, ROC and AUC analyses of these candidate circulating miRNAs revealed strong discriminatory potential between the patient and control groups, indicating their viability as potential biomarkers (Figure 2C and Table III).

Comparison of Circulating miRNA Expression Profiles in the Ruptured and Unruptured Cases

In comparing the RA and UA groups, the expression levels of 14 miRNAs in samples collected on the third day of bleeding, as well as 11 miRNAs from the fifth day, exhibited significant differences. Notably, miRNA expression was downregulated in the RA group relative to the UA group (Figure 3A, 3B). Several miRNAs demonstrated AUC values exceeding 0.8 on the third day, highlighting their effectiveness in distinguishing between patients with unruptured and ruptured aneurysms. These findings indicate that such miRNAs hold considerable promise for accurately differentiating between these two conditions and that alterations in their expression could potentially serve as predictors of aneurysmal rupture (Table IV).

Regarding whether specific miRNAs exhibit significant changes from the third to the fifth day, which may play a role in the progression of aSAH due to complications like vasospasm (that can arise after the fourth day in cases of aneurysm rupture), we compared miRNA expression levels between the two time points in patients with aneurysm rupture. A significant increase was noted in the level of miR-24 on the fifth day compared to the third day after aSAH (FC=1.70, p=0.013), with an AUC value of 0.814 (95% CI: 0.678-0.910; sensitivity=68.00, specificity=88.00, p<0.0001). These results suggest that miR-24 may contribute to the progression of aSAH.

Relationship Between miRNA Expression Patterns and the Clinical Characteristics of Patients with Ruptured Aneurysms

To identify miRNAs that could provide information about the development of ruptured aneurysms and be used as markers, miRNA expression patterns were compared based on the patients' Fisher grades (Grades 1-3 versus 4), Hunt–Hess grades (Grades 1-2 versus 3-5), WFNS grades (Grades 1-2 versus 3-4), and clinical outcomes (mRS scores of 1-2 versus 3-6). No significant difference was observed between miRNA levels and Fisher grades (p>0.05). However, when comparing miRNA levels based on neurological severity, on the third day, the expression of miR-9-3p was significantly reduced in patients with worse neurological conditions (WFNS Grades 3-5) compared to those in better condition (WFNS Grades 1-2; FC=0.53, p=0.047; AUC=0.747, p=0.020; Figure 4A). Similarly, miR-497 expression levels were significantly reduced on the third day in patients with Hunt–Hess Grades 3-5 (FC=0.45, p=0.023; AUC=0.840, p<0.0001; Figure 4B).

Table III: Receiver Operating Characteristic Curve Analysis for Detecting the Effectiveness of microRNAs with Biomarker Potential

Ruptured IA (Day 3 post-SAH) vs Control					**Ruptured IA (Day 5 post-SAH) vs Control**				
miRNA	**Sensitivity (%)**	**Specificity (%)**	**AUC (95% CI)**	**p-value**	**miRNA**	**Sensitivity (%)**	**Specificity (%)**	**AUC (95% CI)**	**p-value**
miR-15a	68.00	100.00	**0.783** (0.602-0.908)	0.0004*	miR-15a	68.00	100.00	**0.846** (0.674-0.948)	<0.0001*
miR-183-5p	84.00	100.00	**0.891** (0.731-0.973)	<0.0001*	miR-183-5p	80.00	100.00	**0.874** (0.709-0.964)	<0.0001*
miR-126	96.00	85.71	**0.903** (0.745-0.979)	<0.0001*	miR-126	72.00	85.71	**0.803** (0.625-0.922)	0.0002*
miR-146-5p	88.00	100.00	**0.914** (0.760-0.984)	<0.0001*	miR-146-5p	80.00	100.00	**0.903** (0.745-0.979)	<0.0001*
miR-502-5p	92.00	71.43	**0.866** (0.699-0.960)	<0.0001*	miR-502-5p	88.00	71.43	**0.820** (0.644-0.933)	0.0006*
miR-221-3p	92.00	85.71	**0.897** (0.738-0.976)	<0.0001*	miR-497	64.00	100.00	**0.840** (0.668-0.945)	<0.0001*
miR-24	88.00	100.00	**0.914** (0.760-0.984)	<0.0001*					
miR-143-3p	84.00	71.43	**0.837** (0.664-0.943)	<0.0001*					
miR-27b-3p	76.00	100.00	**0.834** (0.661-0.942)	<0.0001*					

p value <0.05 is statistically significant,* ***AUC:* *Area under the ROC curve,* ***CI:*** *Confidence interval.*

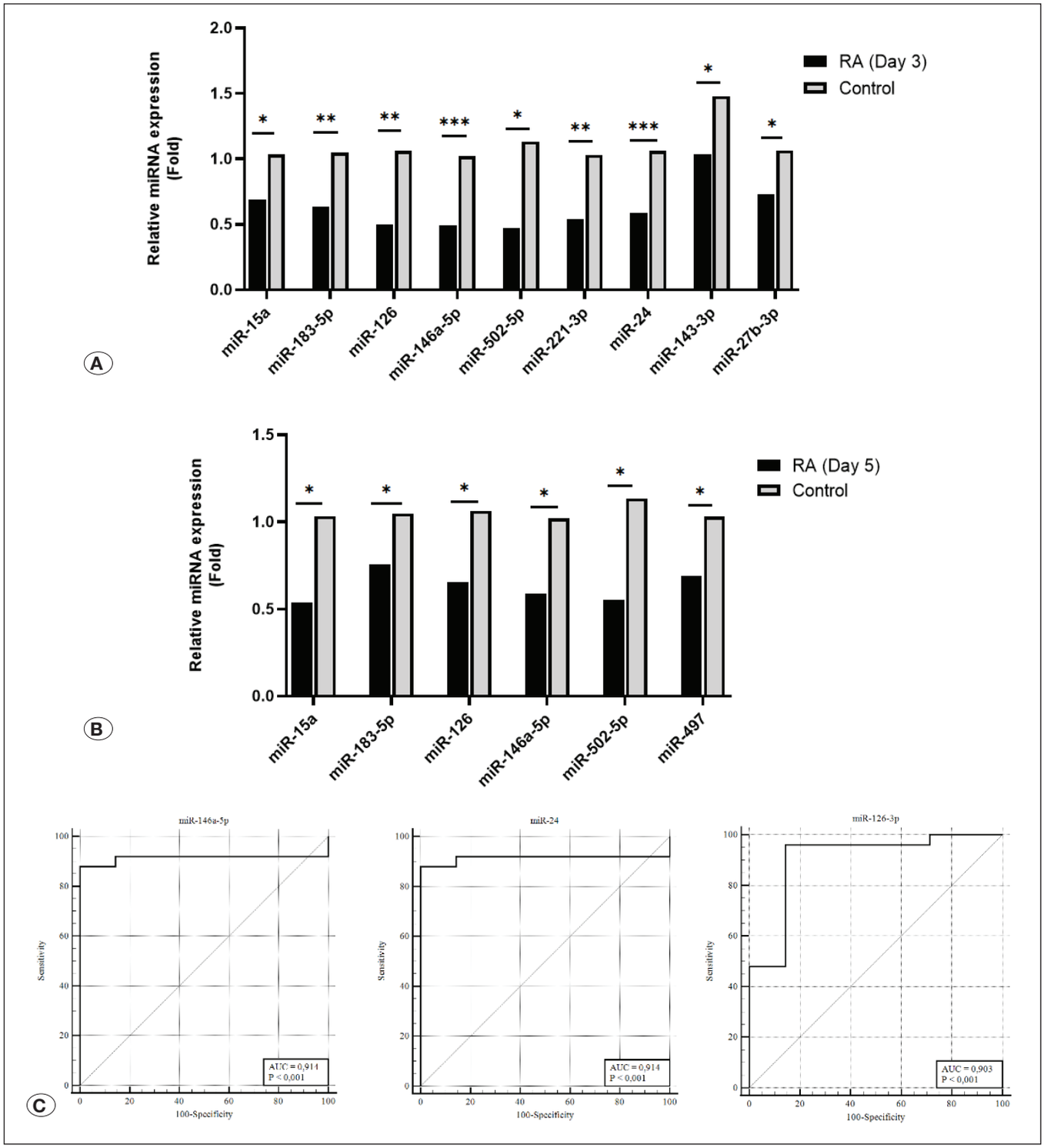


Figure 2: Differential miRNA expression levels in ruptured aneurysm patients versus healthy controls. **A)** Relative expression of miRNAs in ruptured aneurysm patients and controls, presented in bar graphs on a linear scale. miRNA expression levels were significantly downregulated in the RA group compared with controls on Day 3 post-aSAH. **B)** Bar graphs showing six downregulated miRNAs in the RA group compared with controls on Day 5 post-aSAH. Expression levels of all miRNAs are presented as fold changes. Data are shown as means. **C)** ROC curves of patients with ruptured IA and controls. miR-146a-5p, miR-24, and miR-126 achieved AUCs greater than 0.9 on Day 3. Statistically significant differences are marked with asterisks as follows: ***p=0.0001-0.001, **p=0.001-0.01, *p=0.01-0.05. **AUC:** area under the curve, **IA:** Intracranial aneurysm, **RA:** Ruptured aneurysms, **ROC:** Receiver operating characteristic.

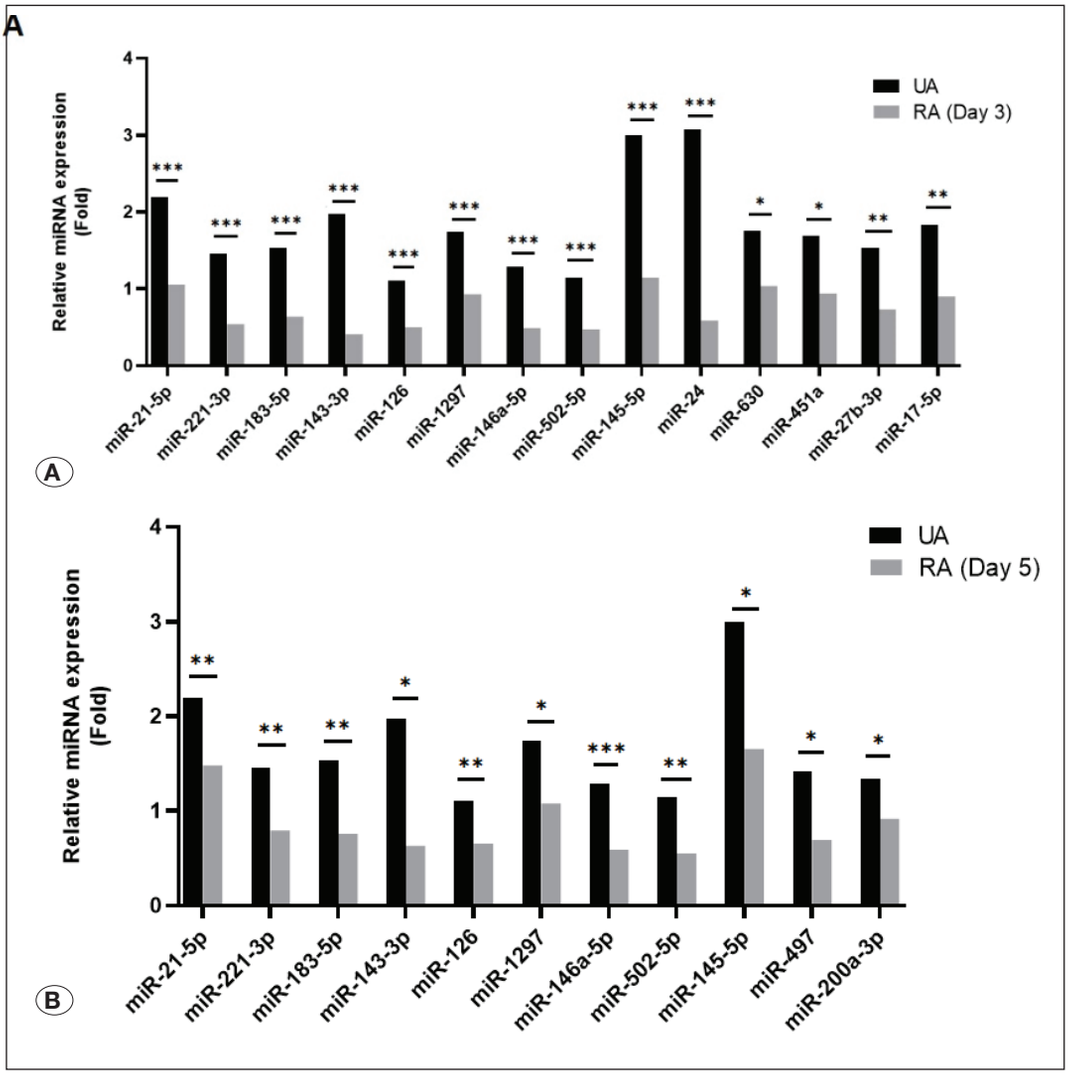


Figure 3: Comparison of the expression levels of differentially expressed miRNAs between groups. **A)** Relative miRNA levels, with a difference between ruptured and unruptured aneurysm cases on Day 3 post-aSAH. **B)** Relative miRNA levels, with a difference between ruptured and unruptured aneurysm cases on Day 5 post-aSAH. miRNA expression levels were significantly downregulated in the RA group compared with the UA group on Days 3 and 5. Statistically significant differences are marked with asterisks as follows: ***p=0.0001-0.001, **p=0.001-0.01, *p=0.01-0.05. **aSAH:** Aneurysmal subarachnoid hemorrhage, **RA:** Ruptured aneurysms, **UA:** Unruptured aneurysms.

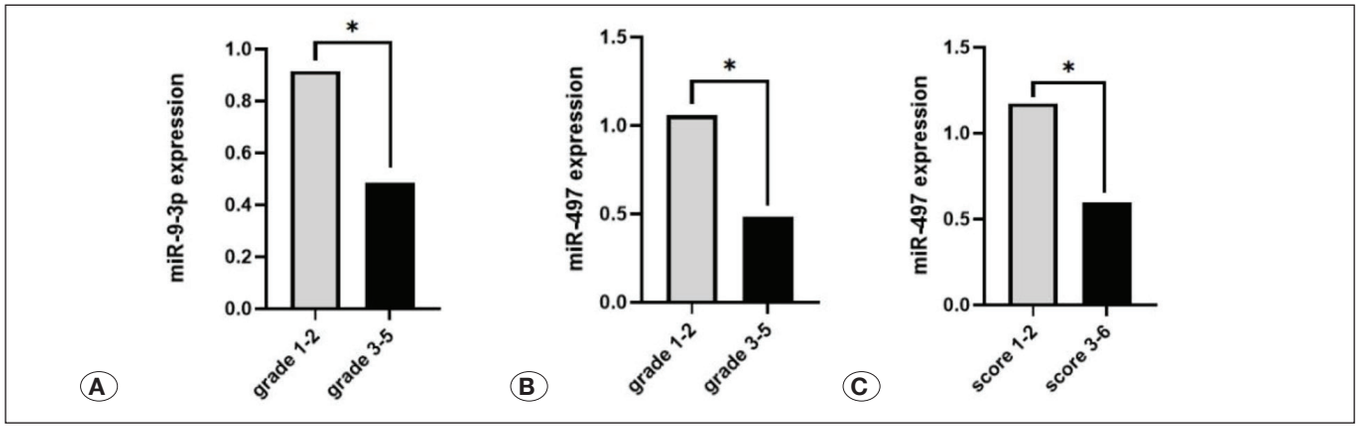


Figure 4: Comparison of miRNA expression in blood with clinical features in aSAH patients. **A)** Relative levels of miR-9-3p in aSAH patients with Hunt–Hess Grades 1-2 and 3-5. **B)** Relative levels of miR-497 in aSAH patients with WFNS Grades 1-2 and 3-5. **C)** Relative levels of miR-497 in aSAH patients with mRS Grades 1-2 (good recovery) and Grades 3-6 (disabled or dead). *A p-value <0.05 is statistically significant. **aSAH:** Aneurysmal subarachnoid hemorrhage, **mRS:** Modified Rankin Scale, **WFNS:** World Federation of Neurosurgical Societies.

Table IV: Receiver Operating Characteristic Curve Analysis for Detecting the Effectiveness of microRNAs with Biomarker Potential in Aneurysmal Rupture

Unruptured vs. Ruptured IA (Day 3 post-SAH)					Unruptured vs. Ruptured IA (Day 5 post-SAH)				
miRNA	**Sensitivity (%)**	**Specificity (%)**	**AUC (95% CI)**	**p-value**	**miRNA**	**Sensitivity (%)**	**Specificity (%)**	**AUC (95% CI)**	**p-value**
miR-21-5p	72.00	76.00	**0.806** (0.670-0.904)	<0.0001*	miR-21-5p	80.00	76.00	**0.771** (0.631-0.878)	0.0001*
miR-221-3p	88.00	96.00	**0.894** (0.775-0.963)	<0.0001*	miR-221-3p	84.00	84.00	**0.849** (0.719-0.934)	<0.0001*
miR-183-5p	84.00	76.00	**0.818** (0.683-0.913)	<0.0001*	miR-183-5p	80.00	76.00	**0.784** (0.645-0.888)	<0.0001*
miR-143-3p	88.00	80.00	**0.878** (0.755-0.954)	<0.0001*	miR-143-3p	72.00	76.00	**0.730** (0.585-0.845)	0.0022*
miR-126	88.00	80.00	**0.875** (0.751-0.952)	<0.0001*	miR-126	60.00	92.00	**0.739** (0.596-0.853)	0.0010*
miR-1297	88.00	72.00	**0.844** (0.714-0.931)	<0.0001*	miR-1297	84.00	68.00	**0.758** (0.616-0.868)	0.0005*
miR-146a-5p	88.00	88.00	**0.882** (0.759-0.956)	<0.0001*	miR-146a-5p	80.00	88.00	**0.851** (0.722-0.936)	<0.0001*
miR-502-5p	88.00	72.00	**0.825** (0.691-0.918)	<0.0001*	miR-502-5p	84.00	72.00	**0.791** (0.653-0.893)	<0.0001*
miR-145-5p	88.00	68.00	**0.789** (0.650-0.891)	<0.0001*	miR-145-5p	60.00	80.00	**0.678** (0.531-0.803)	0.0224*
miR-24	88.00	84.00	**0.867** (0.741-0.947)	<0.0001*	miR-497	72.00	76.00	**0.761** (0.619-0.870)	0.0002*
miR-630	72.00	84.00	**0.744** (0.601-0.857)	0.0010*	miR-200a-3p	84.00	64.00	**0.742** (0.599-0.856)	0.0008*
miR-451a	64.00	80.00	**0.739** (0.596-0.853)	0.0008*					
miR-27b-3p	76.00	76.00	**0.775** (0.635-0.881)	0.0001*					
miR-17-5p	72.00	76.00	**0.757** (0.615-0.867)	0.0003*					

p value <0.05 is statistically significant,* ***AUC:* *Area under the ROC curve,* ***CI:*** *Confidence interval.*

Additionally, patients classified by clinical outcomes were grouped as well-recovered (mRS scores of 1-2) and those who died or were disabled (mRS scores of 3-6). The expression level of miR-497 was found to be significantly lower on the third day in patients with an mRS score of 3-6 compared to those with mRS scores of 1-2 (FC=0.51, p=0.037; AUC=0.753, p=0.015; Figure 4C).

In the group with mRS scores of 3-6, the expression levels of miR-451a, miR-146a-5p, and miR-502-5p (FC=0.65, FC=0.59, and FC=0.47, respectively) were significantly reduced on the fifth day after SAH. The AUC values for miR-451a, miR-146a-5p, and miR-502-5p were 0.753, 0.773, and 0.813, respectively. These data suggest that they can be used as potential biomarkers to predict clinical outcomes in aSAH patients (Figure 5A, 5B).

DISCUSSION

Despite considerable efforts to identify non-genetic factors that could serve as predictors for the risk of IA, clinical studies to date have only identified a few traditional risk factors for unruptured IAs, such as age, gender, smoking, and hypertension (43). Comprehensive data regarding the pathogenesis of IAs remain elusive. Additionally, as the early diagnosis of ruptured IAs, which continue to be a significant cause of morbid-

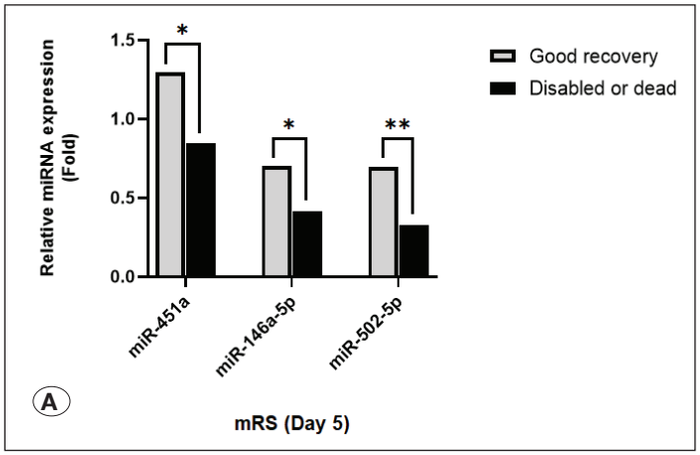
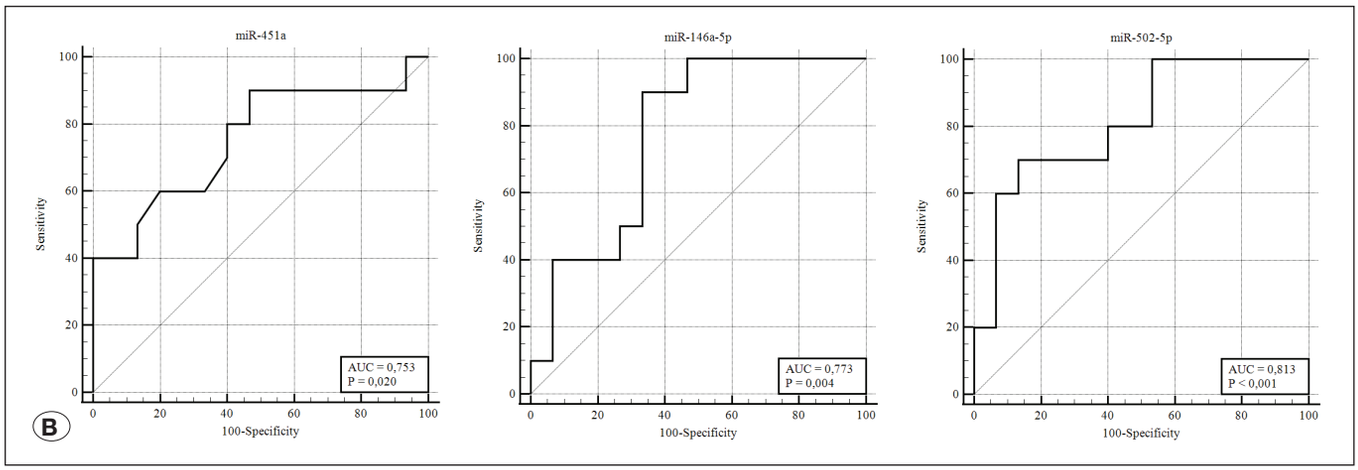


Figure 5: miRNA expression pattern in patients with good and poor clinical outcomes. **A)** Relative levels of miR-451, miR-146a-5p, and miR-502-5p in aSAH patients with good recovery and those who died or became disabled. Expression levels of miR-451, miR-146a-5p, and miR-502-5p were significantly lower in patients with a poor outcome (mRS Grades 3-6) than in those with a good clinical outcome (mRS Grades 1-2) on Day 5 post-SAH. All miRNA levels are presented as fold change. Data are shown as means. **p=0.001-0.01, *p=0.01-0.05. **B)** ROC curve analysis for potential miRNAs and predicted risk of poor outcomes after SAH. miR-502-5p had the strongest predictive value in discriminating patients with good outcomes and those with poor outcomes on Day 5 post-SAH, with an AUC of 0.813, sensitivity of 70%, and specificity of 86.67%. **aSAH:** Aneurysmal subarachnoid hemorrhage, **AUC:** Area under the curve, **mRS:** Modified Rankin Scale, **ROC:** Receiver operating characteristic.



ity and mortality and contribute to the global disease burden, increases the chance of achieving good outcomes, there is a pressing need for easily testable markers that can provide highly accurate information for IA risk, particularly in relation to predicting and prognosticating aSAH (24,37). miRNAs, which are non-coding RNAs, are found in various biological fluids, including plasma, serum, and CSF and are involved in many physiological processes through their roles in gene expression regulation. Therefore, their abnormal expression can often disrupt cellular and biological functions and contribute to the development and progression of disease. Due to their resistance to nucleases and high stability in body fluids (14), miRNAs have been demonstrated to be potential biomarkers for the development of numerous diseases, including cancer, cardiovascular disease, and neurological conditions (9,39). Recently, leveraging these characteristics of miRNAs to identify asymptomatic IAs, predict the risk of ruptured IAs, and provide information about clinical progression has gained importance. A feature of our study is that samples from ruptured cases were collected separately on the third and fifth days of bleeding and analyzed. The aim was to identify miRNAs that may influence the risk of clinical deterioration, such as vasospasm, around the fourth to fifth days post-bleeding.

Research on complications and prognosis following aSAH remains limited. Few studies have examined the relationship between miRNA expression and factors such as vasospasm, delayed cerebral ischemia (DCI), or overall prognosis. Unfortunately, many of these findings lack consistency across different cohorts because of methodological heterogeneities (8).

miRNAs have the potential to serve as prognostic biomarkers for patients with aSAH. Increase in the expression levels of let-7b-5p, miR-19b-3p, miR-125-5p, miR-221-3p, miR-21-5p, and miR-27a-3p in CSF have been associated with a higher risk of delayed cerebral vasospasm (DCV) in SAH patients. Similarly, elevated levels of let-7a-5p, miR-146a-5p, miR-204-5p, miR-221-3p, miR-23a-3p, and miR-497-5p expression in plasma samples taken 3 days post-aSAH have been linked to DCV (31).

miRNAs and their target genes play distinct roles in vascular endothelial inflammation and phenotypic changes of vessel cells. The formation and rupture of IAs have been reported to be associated with endothelial cell dysfunction. Therefore, miRNAs act as epigenetic elements involved in the inflammatory process of aneurysms (44,47).

In a study by Wang et al., the expression profiles of miRNAs in CSF and plasma samples collected from patients with and without DCV were compared 3 and 7 days after aneurysm rupture. Notably, in CSF, miRNAs including let-7b-5p, miR-15b-5p, miR-17-5p, miR-19b-3p, miR-20a-5p, miR-24-3p,

and miR-29a-3p demonstrated an AUC of 1, indicating perfect discrimination. Likewise, let-7a-5p, miR-146a-5p, miR-204-5p, miR-221-3p, miR-23a-3p, and miR-497-5p in plasma achieved AUCs greater than 0.8, demonstrating their strong potential to differentiate between DCV+ and DCV– 3 days after aSAH, even before the onset of DCV events. The authors highlighted their newly designed miRNA panel as a reliable predictor of DCV risk, offering substantial potential for the clinical management of aSAH patients. Previous studies also reported that elevated miR-21-5p and miR-221-3p levels in CSF correlated with DCI, while miR-221-3p, miR-132-3p, and miR-19b-3p in CSF were associated with DCV. Additionally, temporal changes of let-7b-5p and miR-92a-3p in CSF, as well as miR-15a in both the CSF and plasma of DCI patients, have been reported (47).

Jin et al. found that the relative expression of miR-21 was downregulated in the serum specimens of IA patients compared with controls. Of note, the reduction in miR-21 expression was most pronounced in the group with daughter aneurysms. They suggested that the decreased expression of miR-21 in IA cases might offer diagnostic insights regarding aneurysm rupture and could inform clinical intervention (13).

miRNA-21 regulates vascular smooth muscle activity by targeting proteins such as phosphatase and tensin homologous protein (PTEN), Bcl-2, and PDCD-4. When miRNA-21 is expressed at low levels, PTEN expression increases, which reduces the phosphorylation and activation of AKT. This reduction is significant because AKT is a key player in an anti-apoptotic and pro-proliferative pathway that limits IA expansion and the progression of vascular disorders. Recent scientific reports have highlighted the specific role of miR-21 in regulating the biological function of endothelial progenitor cells (EPCs). Studies have shown that exosomes derived from EPCs carry miR-21-5p, which specifically suppresses the expression of thrombospondin 1 (THBS1), an angiogenesis inhibitor, in endothelial cells, thereby facilitating the repair of vascular endothelial cells (10,28). A study conducted by Chen et al. in mouse models revealed that the miR-21 expression level influences the formation and rupture of IA through the JNK signaling pathway-mediated inflammatory response. This finding aligns with our research (4). When the expression of miRNA-21, which acts comprehensively and regulates multiple pathways, decreases, its protective efficacy diminishes, contributing to aneurysm formation due to wall remodeling. Therefore, circulating miRNA-21 expression levels may serve as a useful diagnostic/prognostic biomarker for IAs.

miR-15a plays a significant role in ischemia-induced cerebral vascular endothelial damage, vascular angiogenesis, and endothelial cell proliferation. Previously, increased and decreased expression of miR-15a have been reported to significantly reduce and increase cerebral vascular endothelial cell death due to oxygen-glucose deprivation, respectively. In addition, elevated miR-15a expression may contribute to vascular proliferation or angiogenesis, potentially leading to the development of vasospasm (52,53).

In a study by Supriya et al., miR-15a-5p expression was significantly increased in aSAH cases compared to controls (37). Conversely, Kikkawa et al. (16), in evaluating the chronological changes in miR-15a expression levels in plasma and CSF samples of patients with SAH, found that miR-15a expression decreased from Day 1 to Day 3 in plasma and increased in CSF. In contrast, its expression increased in plasma from Day 5 and remained constant on Day 7. In general, since delayed cerebral ischemia, which is the main cause of clinical deterioration in patients with SAH, mostly occurs between Days 4 and 10 after the onset of SAH, miR-15a expression may also be related to the clinical course of the cases. However, when the clinical scores and miRNA expression of the cases included in this study were compared, no relationship due to miR-15a expression was noted.

Ding et al. studied changes in CSF proteins in aSAH patients using multitargeted Olink proteomics (including a 96-neurology panel and a 96-inflammation panel) to elucidate the pathophysiology of DCI and provide valuable insights into its molecular basis for clinical applications. Based on the neurology panel, they identified several CSF proteins in aSAH patients, mainly MSR1, siglec-1, siglec-9, CTSC, and CTSS. These proteins are involved in defense response regulation, vesicle-mediated transport, and regulation of immune response. Additionally, differentially expressed proteins were notably enriched in various pathways, including the MAPK signaling pathway, RAS signaling pathway, cytokine-cytokine receptor interaction, and lysosome and phagosome pathways. In the inflammation panel, the predominantly identified proteins were IL-6, MCP-1, CXCL10, CXCL-9, and TRAIL, which are involved in the cellular responses to chemokines and chemokine-mediated signaling pathways. Additionally, these differentially expressed proteins were mainly enriched in cytokine-cytokine receptor interactions, viral protein interactions with cytokines and their receptors, chemokine signaling pathways, the NF-κB signaling pathway, and the Toll-like receptor signaling pathway (6). Lu et al. identified a panel of four miRNAs (miR-4532, miR-4463, miR-1290, and miR-4793) that can effectively distinguish SAH patients with DCI from those without DCI, achieving an AUC of 100%. The targets of these miRNAs were found to be enriched in various developmental pathways, including the Wnt, hedgehog, and oxytocin signaling pathways (22).

In our study, we observed a significant decrease in the expression of miR-143 in samples taken on the third day from ruptured cases compared to controls. This finding aligns with previous studies (12,14,36). Recently, the downregulation of miR-143-3p and miR-125b-5p was reported to be associated with worse neurological conditions and the occurrence of vasospasm (8). The downregulation of miR-143 has been identified as a critical step contributing to vascular smooth muscle cells (VSMC) phenotypic modulation, which is essential for cerebral aneurysm formation. In most IA walls, VSMCs migrate to the intima and undergo modulation from a contractile to a synthetic phenotype. This results in their dissociation and leads to myointimal hyperplasia. Several researchers have shown that this process leads to the loss of the structural integrity of the media, the layer that provides support to the vessel wall (1,3,49). Xu et al. reported that both miR-143 and miR-145 interact with the 3'-UTR of KLF5, inhibiting its

post-transcriptional expression (50). Consequently, the downregulation of miR-143/145, combined with the upregulation of KLF5, suggests that VSMC phenotypic modulation significantly influences the formation and growth of IAs. Moreover, NLRP1, a predicted target of miR-143-3p, is a member of the Ced-4 family of apoptosis proteins that can induce caspase-1 activation through the assembly of inflammasomes, which are critical for the production of mature proinflammatory cytokines, including IL-1β and IL-18. IL-18, a predicted target of miR-143-3p, enhances early-stage apoptosis of cultured human umbilical vein endothelial cells and increases vascular endothelial cell death. These interactions suggest that miR-143-3p may play a crucial role in the pathogenesis of IA by regulating apoptosis and inflammation-related pathways (12).

Ryu and colleagues reported that several miRNAs, specifically miR-4732-3p, miR-16-2-3p, miR-6885-3p, miR-29a-3p, miR-148b-3p, miR-374b-5p, and miR-26b-5p, displayed differing levels between patients with and without cerebral vasospasm (CVS). Interestingly, miR-148b-3p was the only differentially expressed miRNA that interacted with rho-associated protein kinase 1 (ROCK1). This interaction is significant, as ROCK1 plays a role in regulating endothelial and vascular tone during vasospasm, and miR-148b-3p was found to be upregulated in patients. The study showed that miR-148b-3p directly interacts with ROCK1, reducing its expression and influencing cell growth, migration, and invasion in human brain endothelial cells through the ROCK-LIMK-cofilin pathway. Moreover, disruption of the ROCK-LIMK-cofilin pathway leads to abnormal organization of the actin cytoskeleton, ultimately causing endothelial cell dysfunction, and may contribute to the development of vasospasm. Notably, cofilin has also been implicated in other forms of brain injury, such as intracerebral hemorrhage and traumatic brain injury, as it influences inflammation and synaptic plasticity. This highlights miR-148b-3p's potential as a promising biomarker and therapeutic target for CVS following aSAH (30).

An in vitro study that simulated the post-SAH extracellular environment highlighted the critical role of exosomal miR-630 in disease pathology. When brain microvascular endothelial cells (BMECs) were exposed to a culture medium containing blood-cerebrospinal fluid, a significant reduction in exosomal miR-630 level was observed, aligning closely with the alterations in CSF exosomal miR-630 in aSAH patients. Additionally, BMECs co-cultured with exosomes transfected with miR-630 mimics exhibited a marked upregulation of ICAM-1, VCAM-1, and the tight junction protein ZO-1 compared to the control group. These findings suggest that exosomal miR-630 may play a pivotal role in modulating cell adhesion and maintaining tight junction integrity in BMECs, potentially contributing to the enhancement of the brain microcirculation (19).

Lopes et al. utilized an NGS platform to analyze global miRNA profiling in whole blood samples from 26 patients, both with and without vasospasm. Their study identified five downregulated miRNAs (miR-7f-5p, miR-126-5p, miR-17-5p, miR-451a, and miR-486-5p) and three upregulated miRNAs (miR-146a-5p, miR-589-5p, and miR-941) that were differentially expressed in aSAH patients compared to controls. Notably, in silico analysis revealed that the THBS1 and VEGFA target genes are associated with aSAH (21). In another study involving serum samples from patients with intracerebral hemorrhage, miR-126 showed a significant decrease in patients compared to controls and was correlated with perihematomal edema. The authors proposed that miR-126 can be used as a potential biomarker for managing perihematomal edema (56). Additionally, Yang et al. (51) found significant alterations in miR-126 levels in patients with IA. Their results showed that both lesion size and miR-126 expression were independent risk factors for aneurysmal rupture, leading them to hypothesize that circulating miR-126 might be a potential diagnostic biomarker for IA occurrence and rupture. Our study also demonstrated that miR-126 expression was significantly decreased in ruptured cases compared to controls, suggesting its potential as an effective marker for distinguishing between ruptured and non-ruptured cases.

Supriya et al. (37) identified five downregulated miRNAs (miR-146a-5p, miR-376c-3p, miR-18b-5p, miR-24-3p, and miR-27b-3p) and three upregulated miRNAs (miR-15a-5p, miR-34a-5p, and miR-374a-5p) that can discriminate between aSAH patients and controls. In addition, circulating levels of miR-146a-5p and miR-27b-3p were associated with the severity of aSAH and patient outcomes. Functional analysis of these differentially expressed miRNAs showed that their target genes were involved in signaling pathways related to inflammation, suggesting they may play critical roles in IA pathology. In a preclinical study, a decreased level of miR-146a was observed in the perihematomal area 48 hours after intracerebral hemorrhage, confirming its anti-inflammatory potential (15).

A PCR array study indicated that circulating miRNA-183-5p, miRNA-let7b-5p, and miRNA-200a-3p were differentially expressed in aSAH patients. Most importantly, elevated levels of miRNA-200a-3p were found exclusively in aSAH patients and not in those with UA, indicating that circulating miR-200a-3p may influence the risk of aneurysmal rupture. Functional analyses of miRNA-183-5p and miRNA-200a-3p showed that their target genes were associated with signaling pathways involved in inflammation and cell proliferation. This evidence suggests that these miRNAs may serve as biomarkers for diagnosing IA and assessing the risk of its subsequent rupture (13,26).

Zheng et al. conducted a comparative analysis of the miRNA expression profiles of peripheral blood samples from patients with aSAH, reporting downregulated expression of miR-23b-3p, miR-590-5p, miR-20b-5p, miR-142-3p, and miR-29b-3p in aSAH patients compared to controls. These miRNAs target genes closely linked to the formation, progression, and rupture of IA, such as TGM2, EREG, EDN1, and COL4A1. Several pathways implicated in SAH were enriched in their analysis, including the Hippo signaling pathway, p53 signaling pathway, cellular senescence, AMPK signaling pathway, focal adhesion, osteoclast differentiation, and the cell cycle. Notably, altered expression of miRNA-23b-3p and miRNA-29b has been demonstrated in IA tissues compared to normal tissues. Additionally, miR-23b is linked to the inflammatory response,

while miR-20b-5p appears to influence the progression of IA by modulating actin cytoskeleton biogenesis. Further research indicates that the decreased expression of miR-590-5p in aSAH enhances angiotensin II-induced endothelial cell apoptosis (54).

Our study also explored the relationships between miRNAs, the severity of aSAH, and the clinical outcomes of the cases. Lai et al. (18) identified significant changes in the expression of miR-502-5p and miR-1297 in aSAH patients. Furthermore, they reported a strong correlation between elevated expression levels of circulating miR-502-5p and poor neurological outcomes. These findings suggest that miR-502-5p may serve as a potential biomarker for the diagnosis and prognosis of aSAH. In another study, Bache et al. (2) analyzed miRNA expression in CSF samples from 63 SAH patients and assessed its relationships with severity and post-SAH clinical outcomes. They found that high levels of miR-9-3p were associated with poor functional outcomes (mRS 3-6) 3 months later. Additionally, they found that high levels of miR-451a were associated with poor neurological status at admission, as measured by the WFNS grade. Two target genes of miR-451a, MMP-2 and MMP-9, are considered significant in the pathogenesis of SAH and its complications due to their roles in the extracellular matrix and immune responses (25,34). Studies on ischemic stroke have also shown that higher levels of miR-9 in CSF or plasma are associated with increased severity and larger infarct volume, suggesting its potential as a future research target (8). The differences in sample sizes make it challenging to directly compare these studies and our findings when assessing the severity of SAH and clinical outcomes.

Our data generally align with those of previous studies. However, small sample sizes, the heterogeneity of the specimens examined, varying sample collection time points, and different miRNA analysis methodologies may have caused discrepancies therein.

We acknowledge some limitations of our current study. First, the small size of our study cohort is an important limitation. Second, the samples could only be collected from RA patients at two time points (3 and 5 days post-SAH). The levels of differentially expressed miRNA in biofluids following SAH can be dynamic (16,29,32,33). Regularly monitoring changes in these miRNA levels could provide more accurate timing for predicting neurological outcomes and preventing complications, thus allowing physicians to adjust therapies for aSAH more effectively. Finally, utilizing additional approaches, such as high-throughput sequencing methods and miRNA-targeted gene expression studies, could help identify novel diagnostic and prognostic biomarkers of IAs and their subsequent rupture.

CONCLUSION

We demonstrated that miRNA expression differences are valuable for distinguishing aSAH cases. In particular, levels of circulating miR-146a-5p, miR-24, and miR-126 measured 72 h after aSAH showed an AUC value greater than 0.9. When comparing the RA and UA groups, several miRNAs were found to be significantly altered in patients with RA. Our results indicate that the levels of these miRNAs on the third day after aSAH can discriminate RA from UA with a higher predicted probability (AUC>0.8) compared to other time points. Additionally, we showed that circulating miR-9-3p and miR-497 have prognostic value for assessing the severity of aSAH, while miR-451a, miR-146a-5p, miR-502-5p, and miR-497 can predict poor clinical outcomes after admission. Future studies are needed to validate these findings in larger cohorts and better understand the specific roles of these miRNAs in the progression and rupture of IAs before they are implemented in clinical practice.

Declarations

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Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Disclosure: The authors declare no competing interests.

Ethical approval: 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 Eskisehir Osmangazi University, Medical Faculty (2023-48). Each participant provided written informed consent.

AUTHORSHIP CONTRIBUTION

Study conception and design: SKA, EEG, EO, SA
Data collection: EO, AOO, OA
Analysis and interpretation of results: SKA, EEG, ES, BDA, SA, EC
Draft manuscript preparation: SKA, EEG, EO
Critical revision of the article: AOO, SA
Other (study supervision, fundings, materials, etc...): SA
All authors (SKA, EEG, EO, OA, EC, ES, BDA, AOO, SA) reviewed the results and approved the final version of the manuscript.

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