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Original Article

Elevated expressions of serum miR-15a, miR-16, and miR-17-5p are associated with acute ischemic stroke

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Abstract: Acute ischemic stroke (AIS) is one of the leading causes of death and disability worldwide. Circulating microRNAs (miRNAs) have been identified as potential biomarkers in the diagnosis and prognosis of multiple human diseases including AIS. In this study, serum expression levels of miR-15a, miR-16, and miR-17-5p were detected in AIS patients (n = 106) and healthy controls (n = 120) using quantitative real-time polymerase chain reaction (qRT-PCR). Statistical analyses were performed to investigate the associations between miRNA levels and AIS risk. The serum expression levels of miR-15a, miR-16, and miR-17-5p increased by 8.3 fold (P = 0.0104), 42 fold (P < 0.0001), and 9.9 fold (P = 0.0002) in AIS patients compared to controls, respectively. Multivariate logistic regression demonstrated that serum miR-17-5p level was a significant and independent predictor for determining the presence of AIS. Receiver operating characteristic (ROC) analysis revealed areas under the curves (AUCs) of 0.698 (95% confidence interval [*CI*]: 0.559-0.837, P = 0.01), 0.82 (95% *CI*: 0.71-0.931, P < 0.001), and 0.784 (95% *CI*: 0.666-0.903, P < 0.001) for miR-15a, miR-16, and miR-17-5p, respectively, while the AUC increased to 0.845 (95% *CI*: 0.74-0.949, P < 0.001) for the combination of these three micoRNAs. Our findings indicate that elevated serum expression of miR-15a, miR-16, and miR-17-5p is strongly associated with AIS and that the combination of these three microRNAs may be a promising serum biomarker for AIS.

Keywords: Acute ischemic stroke (AIS), MiR-15a, MiR-16, MiR-17-5p, Biomarker

Introduction

Stroke is the second leading cause of death worldwide and the leading cause of years of life lost due to premature mortality in East Asia; the predominant type is acute ischemic storke (AIS) [1]. It is estimated that someone has a stroke every 40 s, and a person dies of a stroke every 4 min in the U.S. [2]. The diagnosis of AIS still relies on time-consuming processes including medical history, clinical examination, and neuroimaging [3]. There is a need for rapid, accurate, specific, and sensitive biomarker-based testing to identify AIS patients; such a test might improve risk predication, early diagnosis, and prognostic assessments and could ultimately impact clinical outcomes and quality of life [4]. Although several blood biomarker candidates have been reported to be associated with AIS onset, none has been widely used in clinical practice [5].

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules that play important gene-regulatory roles in plants and animals by pairing with the 3' untranslated regions (3'UTRs) of specific protein-coding genes to repress their translation or induce their degradation [6]. It is estimated that more than one-third of human genes are conserved miRNA targets [7]. MiRNAs play key regulatory roles in various physiological processes and the pathogeneses of several diseases including cancer [8]. Recent studies have demonstrated altered miRNA expression profiles in AIS patients [9] and rodent models [10]. Specific serum miRNAs might serve as potential biomarkers of AIS [11]; for example, miR-15a and miR-16 are increased in the serum of patients with critical limb ischemia [12], and miR-15a was identified as a key factor of both myocardial ischemia [13] and ischemic stroke [14]. In addition, miR-17-5p may be a critical

Table 1. Baseline participant characteristics

Characteristic	Control *n* = 120	AIS *n* = 106	*P*
Age (years)	61.5 ± 9.0	64.8 ± 11.1	0.224
Male sex n (%)	58 (48.3)	55 (51.9)	0.085
BMI (kg/m^2)	23.5 ± 3.6	23.9 ± 3.0	0.723
Smoking n (%)	25 (20.8)	36 (34.0)	0.203
Drinking n (%)	54 (45.0)	51 (48.1)	0.862
Hypertension n (%)	43 (35.8)	64 (60.4)	0.000
Diabetes mellitus n (%)	38 (31.7)	55 (51.9)	0.002
Total cholesterol (mmol/L)	4.61 ± 1.15	4.62 ± 1.04	0.964
Triglycerides (mmol/L)	1.24 (0.83, 1.85)	1.31 (0.94, 1.99)	0.493
LDL (mmol/L)	2.83 ± 0.90	2.93 ± 0.95	0.706
HDL (mmol/L)	1.23 ± 0.39	0.99 ± 0.20	0.009
ApoA1 (g/L)	1.47 (1.17, 1.68)	1.14 (1.0, 1.41)	0.013
ApoB (g/L)	0.78 ± 0.29	0.96 ± 0.23	0.030
Lpa (g/L)	0.14 (0.07, 0.54)	0.22 (0.12, 0.46)	0.501

AIS, acute ischemic stroke; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lpa, lipoprotein(a). Data are presented as mean ± standard deviation or medians (25th percentile, 75th percentile).

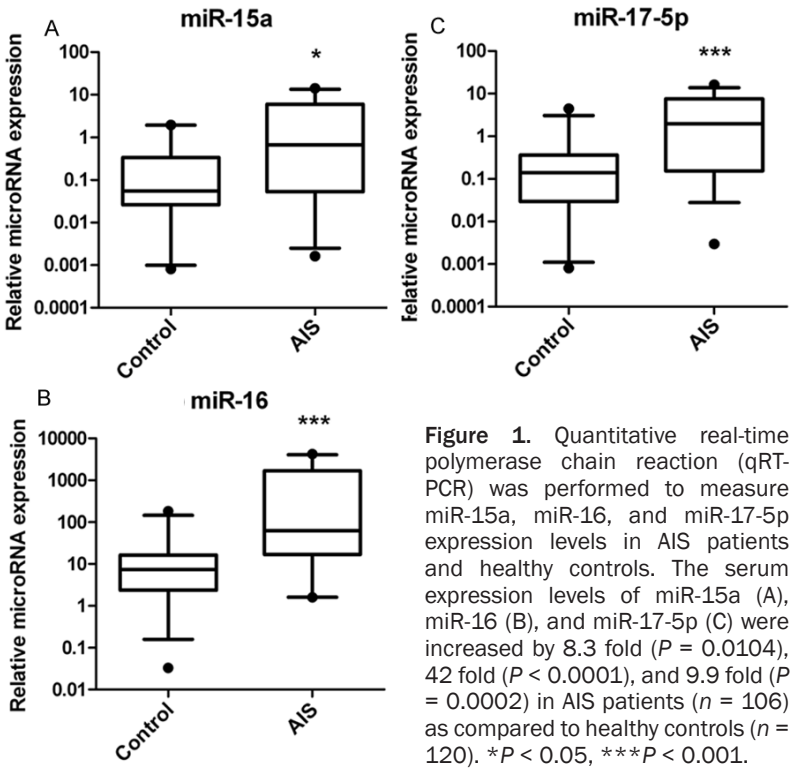

Figure 1. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to measure miR-15a, miR-16, and miR-17-5p expression levels in AIS patients and healthy controls. The serum expression levels of miR-15a (A), miR-16 (B), and miR-17-5p (C) were increased by 8.3 fold ($P = 0.0104$), 42 fold ($P < 0.0001$), and 9.9 fold ($P = 0.0002$) in AIS patients ($n = 106$) as compared to healthy controls ($n = 120$). *$P < 0.05$, ***$P < 0.001$.

factor in post-stroke adult neurogenesis [15]. Taking these findings together, we hypothesized that the combination of miR-15a, miR-16, and miR-17-5p may be a useful AIS biomarker.

The aim of this study was to investigate the expressions of miR-15, miR-16, and miR-17-5p in the peripheral blood of AIS patients and healthy controls to evaluate the utility of these three miRNAs as AIS serum biomarkers.

Subjects and methods

Subject recruitment and sample collection

A total of 106 AIS patients admitted to Emergency Department of the Second Affiliated Hospital of Nanjing Medical University between August 2012 and February 2015 were enrolled in this study. The cohort included 55 men and 51 women with a mean age of 64.8 years (range, 39-88 years). AIS was confirmed with brain computed tomography (CT) or magnetic resonance imaging (MRI). Patients with the following disorders were excluded from the study: (1) symptoms indicative of subarachnoid hemorrhage, even if no imaging findings of hemorrhage were found on CT or MRI; (2) intracranial hemorrhage; (3) acute myocardial infarction; and (4) critical limb ischemia. Age- and sex-matched healthy controls without symptoms of AIS and history of cerebrovascular diseases were recruited from the physical examination center.

Clinical characteristics and biochemical parameters

Peripheral blood samples were collected from all participants before treatment and transferred to separation gel vacuum procoagulant collective tubes. Serum samples were collect-

Table 2. Correlations between miR-15a levels and clinical parameters

Characteristic	Correlation coefficient (r)	*P*
Age (years)	0.276	0.040
BMI (kg/m^2)	0.307	0.057
Total cholesterol (mg/dL)	0.032	0.821
Triglycerides (mg/dL)	-0.091	0.515
LDL (mg/dL)	0.119	0.399
HDL (mg/dL)	-0.250	0.074
ApoA1 (g/L)	-0.171	0.263
ApoB (g/L)	0.204	0.179
Lpa (g/L)	0.001	0.996

ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lpa, lipoprotein(a).

Table 3. Correlation between miR-16 levels and clinical parameters

Characteristic	Correlation coefficient (r)	*P*
Age (years)	0.047	0.732
BMI (kg/m^2)	0.065	0.693
Total cholesterol (mg/dL)	-0.076	0.589
Triglycerides (mg/dL)	-0.031	0.826
LDL (mg/dL)	-0.067	0.636
HDL (mg/dL)	-0.376	0.006
ApoA1 (g/L)	-0.301	0.044
ApoB (g/L)	0.233	0.123
Lpa (g/L)	-0.109	0.489

ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lpa, lipoprotein(a).

ed by centrifugation at 3500 r/min for 10 min and stored at -70°C for subsequent experiments. Total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), and lipoprotein a (Lpa) were measured from samples collected after at least 12 h of fasting, using the Toshiba 200FR Neo chemistry autoanalyser (Toshiba Medical Systems, Tokyo, Japan). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Table 4. Correlation between miR-17-5p levels and clinical parameters

Characteristic	Correlation coefficient (r)	*P*
Age (years)	0.101	0.461
BMI (kg/m^2)	-0.179	0.277
Total cholesterol (mg/dL)	0.097	0.491
Triglycerides (mg/dL)	0.234	0.091
LDL (mg/dL)	-0.022	0.874
HDL (mg/dL)	-0.207	0.140
ApoA1 (g/L)	-0.077	0.614
ApoB (g/L)	0.154	0.314
Lpa (g/L)	0.079	0.617

ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Lpa, lipoprotein(a).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from serum samples using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Two chloroform purification steps were performed to remove proteins. RNA concentration and purity were determined with a NanoDrop™ ND-2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA quality was assessed using denaturing gels (15% polyacrylamide and 1% agarose).

The miRNAs assessed in the present study included hsa-miR-15a (miRBase aAccession number: MIMAT0000068), hsa-miR-16 (miRBase accession number: MIMAT0000069), and has-miR-17-5p (miRBase accession number: MIMAT0000070). The expression level of each miRNA relative to U6 snRNA was confirmed. Total RNA (1000 ng) was reverse-transcribed to cDNA using TaqMan microRNA RT Kit and stem-loop RT primers (Applied Biosystems, Foster City, CA, USA) with a final volume of 20 μL (added to 5 μL double-distilled water) according to the manufacturer's protocol. QRT-PCR was performed with 1 μL reverse-transcribed product added into each well of a 384-well optical plate using the TaqMan PCR kit on the ABI 7900 Real-Time PCR System (Applied Biosystems). The reaction process was initiated at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min as described

Table 5. Logistic regression analysis for presence of AIS in participants

	Univariate logistic regression			Multivariate logistic regression		
	OR	95% CI	*P*	OR	95% CI	*P*
Hypertension	6.900	2.10-22.57	0.001	34.200	1.845-634.1	0.018
Diabetes mellitus	7.527	2.21-25.60	0.001	19.820	1.27-351.61	0.033
HDL (mg/dL)	0.035	0.003-0.425	0.009	0.0250	0.00-4.82	0.17
ApoA1 (g/L)	0.730	0.172-5.155	0.936	-	-	-
ApoB (g/L)	26.863	1.428-505.12	0.028	5.328	0.008-3424	0.612
miR-15a	1.710	1.021-2.866	0.042	1.314	0.344-5.018	0.952
miR-16	1.013	0.999-1.027	0.077	-	-	-
miR-17-5p	1.943	1.139-3.313	0.015	3.968	1.001-14.29	0.035

ApoA1, apolipoprotein A1; ApoB, apolipoprotein B CI, confidence interval; HDL, high-density lipoprotein; OR, odds ratio.

in the protocol. An equal number of samples from AIS patients and controls were arranged on each plate. All reactions were performed in triplicate. After the reactions, the Ct values were determined using the fixed threshold settings, and mean Ct was estimated from triplicate PCR results. The expression levels of the three target miRNAs relative to U6 snRNA were determined using the $2^{-\Delta Ct}$ method.

Ethical consideration

This study was approved by the Institutional Ethics Committee of the Second Affiliated Hospital of Nanjing Medical University and the Institutional Review Board of Nanjing Medical University. All procedures involved in human subjects were managed in accordance with national law and regulation and the Helsinki Declaration. Written informed consent was obtained from all participants after they received a detailed description of the potential benefits of the study.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (version 19.0, IBM Corp., Armonk, NY, USA). Normally distributed variables are expressed as mean ± standard deviation (SD), non-normally distributed variables are expressed as medians (25^{th}, 75^{th} percentiles), and categorical variables are expressed as number (%). All miRNA expression levels were non-normally distributed. Mann-Whitney U tests were used to compare cases and controls. Comparisons for all proportions were performed using Pearson's χ^2 test. Spearman correlation coefficients were calculated to determine associations between miRNA levels and various laboratory markers. We used unconditional logistic regression analyses to assess the association between AIS and the miRNAs while simultaneously controlling for multiple covariates including age, sex, BMI, smoking, alcohol consumption, hypertension, diabetes mellitus, and hyperlipidemia. Receiver operating characteristic (ROC) curves were plotted for each miRNA and the combination of the three miRNAs. All tests were two-sided, and significance was set at $P < 0.05$.

Results

Baseline participant characteristics

The baseline characteristics of the 226 participants are listed in **Table 1**. There was no difference in age, BMI, sex ratio, smoking, or drinking between the two groups. The proportions of hypertension and diabetes mellitus were higher in the AIS group. Among the laboratory markers, AIS patients had higher levels of ApoB and lower levels of HDL and ApoA1. Total cholesterol, triglycerides, LDL, and Lpa did not differ between the two groups.

Serum miR-15a, miR-16, and miR-17-5p levels

Serum levels of miR-15a, miR-16, and miR-17-5p were significantly higher in AIS patients compared to control subjects (**Figure 1**). The expression of miR-15a, miR-16, and miR-17-5p were increased by 8.3 fold ($P = 0.0104$), 42 fold ($P < 0.0001$), and 9.9 fold ($P = 0.0002$) in the serum of AIS patients relative to controls.

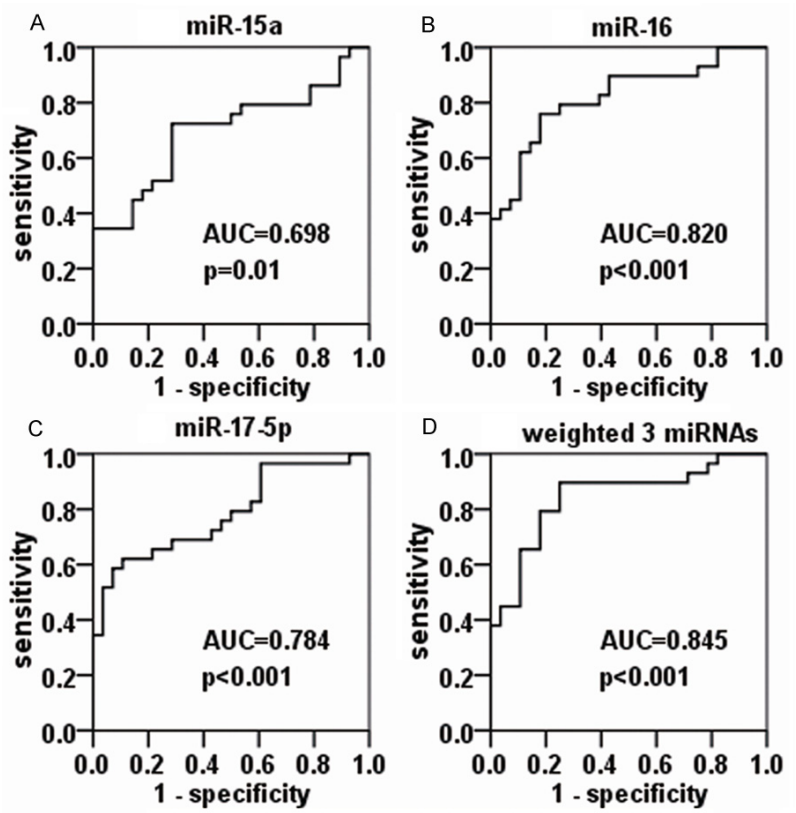

Figure 2. Receiver operating characteristic (ROC) analysis of miR-15a, miR-16, and miR-17-5p for AIS. The areas under curve (AUCs) are 0.698 (95% *CI*: 0.559-0.837, *P* = 0.01), 0.82 (95% *CI*: 0.71-0.931, *P* < 0.001), and 0.784 (95% *CI*: 0.666-0.903, *P* < 0.001) for miR-15a (A), miR-16 (B), and miR-17-5p (C), respectively, and the AUC increases to 0.845 (95% *CI*: 0.740-0.949, *P* < 0.001) for the combination of all three miRNAs (D).

Association of serum miRNA expression levels with clinical characteristics

Serum miR-15a levels showed a significant positive correlation with age (r = 0.276, *P* < 0.05; **Table 2**). There was a strong negative correlation between serum miR-16 levels and HDL (r = -0.376, *P* < 0.01) and ApoA1 (r = -0.301, *P* < 0.05) (**Table 3**). No significant correlation was observed between miR-17-5p and any clinical characteristic (**Table 4**).

Logistic regression analysis

Simple logistic regression analysis revealed that hypertension; diabetes mellitus; and HDL, ApoA1, and miR-15a, miR-16, and miR-17-5p levels were associated with the presence of AIS as diagnosed by the CT or MRI. These variables were entered into a backward, stepwise, multivariate logistic regression model. The results demonstrated that serum miR-17-5p level was a significant and independent predictor for AIS (**Table 5**).

ROC analysis

ROC analysis was performed to evaluate whether the tested serum miRNAs were useful AIS biomarkers (**Figure 2**). The area under curve (AUC) was 0.698 (95% confidence interval [*CI*]: 0.559-0.837, *P* = 0.01), 0.82 (95% *CI*: 0.71-0.931, *P* < 0.001) and 0.784 (95% *CI*: 0.666-0.903, *P* < 0.001) for miR-15a, miR-16, and miR-17-5p, respectively, while the AUC increased to 0.845 (95% *CI*: 0.74-0.949, *P* < 0.001) for the combination of all three miRNAs.

Discussion

Circulating miRNAs have been identified as potential biomarkers for multiple conditions including cancer [16, 17] and cardiovascular and cerebrovascular diseases [18, 19]. The results of the present study demonstrate elevated serum levels of miR-15a, miR-16, and miR-17-5p in AIS patients compared to the controls. Moreover, miR-17-5p was an independent predictor for the presence of AIS. An ROC analysis revealed that the combination of miR-15a, miR-16, and miR-17-5p may be a potential AIS biomarker.

Both miR-15a and miR-16 are localized in the minimally deleted region at chromosome 13q14 and are highly expressed in $CD5^+$ B cells [20]. Previously, miR-15a and miR-16 were predominantly studied in chronic lymphocytic leukemia (CLL) [21, 22] and tumors [23, 24]. MiR-15a and miR-16 act as tumor suppressor genes in pituitary tumors by directly targeting *Sox5*, imply that these miRNAs have potential as therapeutic targets for invasive pituitary tumors [25]. miR-15a and miR-16 induce apoptosis by targeting Bcl2 and control cell proliferation by

targeting Akt3, nuclear factor kappa B (NF-κB), and various cell cycle regulators such as cyclins D1 (CCND1) and D2 (CCND2) [26]. Recent studies have shown miR-15a and miR-16 suppress angiogenesis by targeting vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)-2 [26, 27]. Gao et al. reported that miR-15a plays a significant role in the regulation of neuronal maturation, and its overexpression inhibits dendritic morphogenesis in immature neurons [28]. In addition, these two miRNAs have been demonstrated to play important roles in limb ischemia [12]. miR-15a was recently identified as a key factor involved in myocardial ischemia [13] and ischemic stroke [14]. Yin et al. reported that oxygen-glucose deprivation may activate miR-15a expression in cerebral vascular endothelial cells; decreases in miR-15a expression may protect against blood-brain barrier disruption and cerebral infarction in mice after transient focal cerebral ischemia [14]. These findings suggest that miR-15a may be a viable therapeutic target in addition to a diagnostic tool.

Results obtained in the last decade suggest that miR-17-5p could be a diagnostic biomarkers for breast cancer [29]. It was recently shown that miR-17-5p has more complex functions in cell cycling than miR-15a and miR-16 [30-32], and the miR17-92 cluster may be a critical factor in post-stroke adult neurogenesis [15]. Hong et al. showed that a synthetic miR-17-5p mimic is able to rescue the proliferation defect of Dicer1-null astrocytes, while an antisense inhibitor of miR-17-5p blocks lipopolysaccharide-induced astrocytic proliferation [33]. In this case-control study, we observed a significant positive association between serum miR-17-5p levels and the risk of AIS that persisted after adjusting for various AIS risk factors in multivariate models. This result suggests that miR-17-5p might be an independent predictor for AIS. Chen et al. reported that miR-17-5p may be a circulating biomarker for coronary atherosclerosis severity in patients with coronary artery disease [34]. We did not observe a significant correlation between miR-17-5p and serum lipid levels in the present study.

Individual miRNAs have been identified as biomarkers of AIS; however, they have not shown satisfactory sensitivity or specificity. MiR-15a, miR16-1, and the miR-17-92 cluster play important regulatory roles in cell proliferation, differentiation, and apoptosis. High levels of miR-15a, miR-16-1, and miR-17 are associated with shorter progression-free survival (PFS) in multiple myeloma, suggesting poor prognosis [35]. Integration of multiple miRNAs as blood biomarkers would be useful for the diagnosis, treatment, and management of AIS [36]. MiR-15a and miR-16 may influence angiogenesis, whereas miR-17-5p could affect neurogenesis, two critical processes for repairing cerebral ischemia injury. We observed higher levels of miR-15a, miR-16, and miR-17-5p in AIS patients and found that the combination of these three miRNAs showed great diagnostic value.

It is important to consider our findings in the context of several limitations. Firstly, the biological functions of miR-15a, miR-16 and miR-17-5p were not investigated as we did not examine their target genes. The biological function of an individual miRNA varies greatly because it can target multiple mRNA sites. Although a large number of miR-15a/16 and miR-17-5p target genes were identified previously, their exact roles in the brain after AIS onset remain to be clarified. Secondly, whether the expressions of miR-15a, miR-16, and miR-17-5p undergo dynamic changes requires further investigation and we did not perform any analyses to determine whether miRNA levels could be used to predict outcome. Thirdly, we assessed a relatively small number of subjects.

Conclusions

In conclusion, our findings show up-regulation of serum miR-15a, miR-16, and miR-17-5p levels in AIS patients compared to healthy controls. MiR-17-5p was an independent predictor of AIS. The findings suggest that the combination of miR-15a, miR-16, and miR-17-5p may be a promising AIS biomarker. Repeated miRNAs measurements in a larger-scale study and mechanistic experiments into an association between AIS and these three miRNAs are warranted to confirm their utility as biomarkers.

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Disclosure of conflict of interest

None.

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