Altered MicroRNA Expression in Intracranial Aneurysmal Tissues: Possible Role in TGF-β Signaling Pathway

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Abstract

The molecular mechanisms behind the rupture of intracranial aneurysms remain obscure. MiRNAs are key regulators of a wide array of biological processes altering protein synthesis by binding to target mRNAs. However, variations in miRNA levels in ruptured aneurysmal wall have not been completely examined. We hypothesized that altered miRNA signature in aneurysmal tissues could potentially provide insight into aneurysm pathophysiology. Using a high-throughput miRNA microarray screening approach, we compared the miRNA expression pattern in aneurysm tissues obtained during surgery from patients with aneurysmal subarachnoid hemorrhage (aSAH) with control tissues (GEO accession number GSE161870). We found that the expression of 70 miRNAs was altered. Expressions of the top 10 miRNA were validated, by qRT-PCR and results were correlated with clinical characteristics of aSAH patients. The level of 10 miRNAs (miR-24-3p, miR-26b-5p, miR-27b-3p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-193a-3p, miR-199a-5p, miR-365a-3p/365b-3p, and miR-497-5p) was significantly decreased in patients compared to controls. Expression of miR-125b-5p, miR-143-3p and miR-199a-5p was significantly decreased in patients with poor prognosis and vasospasm. The target genes of few miRNAs were enriched in Transforming growth factor-beta (TGF-β) and Mitogen-activated protein kinases (MAPK) pathways. We found significant negative correlation between the miRNA and mRNA expression (TGF-β1, TGF-β2, SMAD family member 2 (SMAD2), SMAD family member 4 (SMAD4), MAPK1 and MAPK3) in aneurysm tissues. We suggest that miR-26b, miR-199a, miR-497and miR-365, could target multiple genes in TGF-β and MAPK signaling cascades to influence inflammatory processes, extracellular matrix and vascular smooth muscle cell degradation and apoptosis, and ultimately cause vessel wall degradation and rupture.

Keywords Intracranial aneurysm · Aneurysmal subarachnoid haemorrhage · microRNA · Transforming growth factor-beta · Mitogen-activated protein kinases

Abbreviations

aSAH Aneurysmal subarachnoid haemorrhage
ELISA Enzyme-linked immunosorbent assay
GCS Glasgow coma scale
GO Gene ontology
IA Intracranial aneurysm
KEGG Kyoto encyclopedia of genes and genomes
MAPK Mitogen-activated protein kinases
MiRNA Micro RNA
qRT-PCR Quantitative real time polymerase chain reaction
SMAD2 SMAD family member 2
SMAD4 SMAD family member 4
TGF-β Transforming growth factor-beta
WFNS World federation of neurological surgeons

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Introduction

Intracranial aneurysms (IAs) represent pathological focal dilations of the wall of cerebral arteries (Chalouhi et al. 2013). Most IAs are clinically silent during the course of an individual’s lifetime. A few may become symptomatic, usually due to its rupture, and present as aneurysmal subarachnoid haemorrhage (aSAH) (Feigin et al. 2005). Aneurysmal subarachnoid haemorrhage is a catastrophic event resulting in a high rate of death (50%) and disability (60%) (D’Souza 2015; Zacharia et al. 2010). The exact cellular and molecular mechanism of IA formation, growth, and rupture are poorly understood. The interplay of an array of genetic and environmental factors is believed to increase the risk for aSAH (Steiner et al. 2013; Frosen et al. 2012). Further, several factors that influence vascular remodeling and contribute to the structural integrity of the blood vessels may participate in the aneurysm pathogenesis (Cahill and Redmond 2016).

MicroRNAs (miRNAs) are short, endogenous non-coding RNA molecules that influence gene expression by binding to the target mRNAs and interfering with the translational machinery thereby altering protein synthesis (Bartel 2004; Ambros 2004). Emerging data suggest that miRNAs are amply present in vascular tissue and play a significant role in vascular integrity (Jamaluddin et al. 2011). Further, the contribution of miRNAs in regulating proliferation, migration, and inflammatory response in vascular endothelial and smooth muscle cells have been reported, indicating that their aberrant function could potentially influence the development of vascular disease (Bartel 2004; Karp and Ambros 2005). A few studies have reported altered miRNA profiles in biofluids of aSAH patients (Meeuwsen et al. 2017; Jin et al. 2013; Li et al. 2014; Supriya and Christopher 2020; Stylli et al. 2017). However, there is a lack of reports on miRNA expression in aneurysm tissues, and their role in aneurysm pathogenesis is not clearly understood. We hypothesized that altered miRNA signature in intracranial aneurysmal tissues could provide insight into aneurysm pathophysiology. Therefore, using a high throughput miRNA microarray screening approach, we aimed to identify the dysregulated miRNA in aneurysm tissue obtained from aSAH patients during surgery, detect the putative gene targets of the dysregulated tissue miRNAs and elucidate their possible signaling pathways.

Materials and Methods

Patient and Tissue Samples

This case–control study was approved by the Institutional Ethics Committee. Written informed consent was taken from all study participants or first-degree relatives. Full-thickness vessel walls were collected from 29 aSAH patients undergoing direct microsurgical aneurysm repair of ruptured IAs at our centre. The diagnosis of aSAH was established by clinical examination and brain imaging techniques. For the control group, intercostal arteries were obtained from 20, age- and sex-matched patients undergoing intercostomuscucutaneous nerve transfer for brachial plexus injury (Table 1). Tissue samples were snap-frozen in liquid nitrogen immediately after resection and kept in RNA later (Sigma Aldrich, USA), a RNA stabilization solution, and stored at − 80 °C till analysis.

Clinical Assessment

After clinical examination, the neurological status was graded as per the World Federation of Neurological Surgeons (WFNS) grading system (WFNS grades I to V) using the Glasgow coma scales (GCS) (Rosen and Macdonald

Table 1  General characteristics of controls and patients with aSAH

| Parameters | Controls | Patients | P value* |
|------------|----------|----------|----------|
| Age, years (min–max) | 48.30 ± 4.07 (39–79) | 52.38 ± 2.29 (30–75) | 0.0839 |
| Sex, male/female | 12/8 | 19/10 | 0.767 |
| Hypertension, n (%) | 4 (20) | 15 (51.72) | 0.037 |
| Diabetes, n (%) | 2 (10) | 3 (10.34) | 1.000 |
| Smoking, n (%) | 4 (20) | 8 (27.58) | 0.737 |
| Alcohol, n (%) | 3 (15) | 7 (24.13) | 0.495 |

*P value < 0.05 is statistically significant

Hypertension was defined as diastolic blood pressure (DBP) ≥ 90 mm Hg and/or systolic blood pressure (SBP) ≥ 140 mm Hg and/or use of antihypertensive medication. Diabetes mellitus was defined as venous plasma glucose concentration of ≥ 126 mg/dl after an overnight fast and/or ≥ 200 mg/dl, 2 h after a meal, or the use of insulin or oral hypoglycemic agents. A smoker was defined as a person smoking at least one cigarette per day. Alcoholics are defined as more than 14 drinks per week.
Further, based on the volume of blood observed on the brain CT scan, the severity of aSAH was categorized according to the Fisher grading system (Fisher grades I to IV) (Fisher et al. 1980). Patients were followed up after discharge to identify complications like hydrocephalus and vasospasm. The outcome of the patients at the time of discharge was assessed using the GCS (Jennett and Bond 1975).

RNA Extraction and Quality Analysis

Total RNA was extracted from tissue samples using TRIzol Reagent (Sigma Aldrich, USA) and then purified using the RNeasy mini kit (QIAGEN, Germany), following the kit protocol. RNA concentrations and purity were examined in each sample by a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, USA), and a 260/280 ratio between 1.8 and 2.1 was considered acceptable for further analysis. The integrity of total RNA and the presence of DNA as a contaminant were determined on denaturing agarose gel stained with ethidium bromide and analysis of the intensity of 28S/18S bands in ~2:1 ratio. The integrity and quality of tissue RNA samples were further analyzed using a total RNA chip on Agilent 2100 Bioanalyzer (Agilent Technologies, USA) according to the manufacturer’s instructions to ensure RIN integrity number > 7.

MiRNA Microarray Profiling

To identify differentially expressed miRNA in aneurysm tissues, miRNA profiling was carried out from total RNA pooled in equimolar amounts from each sample (8 aneurysm walls and 8 control tissues in technical replicates) using miRCURY LNA™ miRNA Array 7th generation (Exiqon, Denmark) which comprises of control probes to assure optimal labelling and hybridization (52 different RNA spike-in controls), and 3100 capture probes for human miRNAs annotated in miRBase 19.0. Sample labelling and hybridization were carried out according to the kit protocol. Briefly, total RNA from both sample and reference was labelled with Hy3™ and Hy5™ fluorescent labels, respectively, and hybridized on the array using a Tecan HS4800™ hybridization station. After hybridization, miRNA array slides were scanned in an Agilent G2565BA Microarray Scanner System (Agilent Technologies, USA) and analysis was performed using ImaGeneR 9 (miRCURY LNA™ miRNA Array Analysis Software, Exiqon, Denmark). Quantified signals of the duplicate spots of each miRNA were normalized using the global Lowess (Locally Weighted Scatter Plot Smoothing) regression algorithm method. The microarray data discussed in this study have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are available through GEO Series accession number GSE161870.

Quantitative (RT-qPCR) of Selected miRNA and mRNA Targets

For miRNA amplification, reverse transcription of enriched short RNA was conducted using miRCURY LNA™ Universal cDNA synthesis kit (Exiqon, Denmark) with UniSp6 (spike-in control) as an internal control following the manufacturer’s directives. Quantitative PCR assay was done in technical replicates (a repeated measure of the same biological samples) using miRNA LNA™ PCR primer sets (Exiqon, Denmark) and Exi-LENT SYBR® Green master mix (Exiqon, Denmark) according to the manufacturer’s instructions on 7500 fast real-time PCR system (Applied Biosystems, USA). The targeted miRNA–specific LNA primer list for qPCR reactions is given in Supplementary Table 1 in ESM_2.

For mRNA targets, cDNA was synthesized from total RNA with the high capacity cDNA reverse transcription kit (Applied Biosystems, USA). Reverse transcription was conducted in accordance with the manufacturer’s protocol using random primers and up to 100 ng of total RNA for each sample. TaqMan qPCR amplification of selected genes was achieved using TaqMan Universal Master Mix and custom-designed TaqMan gene expression assay probe (Applied Biosystems, USA) following the manufacturer’s protocol. The lists of genes selected for the study are shown in Supplementary Table 2 in ESM_2. The miRNA expression was normalized to miR-150, the most stably expressed miRNA (endogenous control) ranked by NormFinder algorithm (D’Haene et al. 2012) and mRNA to GAPDH expression, to calculate $-\Delta\Delta Ct$ value. The $-\Delta\Delta Ct$ value was then calculated by subtracting the $-\Delta Ct$ value of pooled control sample (measured by qPCR) from the $-\Delta Ct$ value of aSAH patients and control samples. The miRNA and mRNA expression were normalized by the $2^{-\Delta\Delta Ct}$ method. Gene and miRNA expression data were represented as fold change and log2 fold change, respectively (The miRNA values were base-2 log-transformed to normalize the results with equal values, for statistical analysis and graph representation).

Functional Analysis of Targeted miRNAs

Pathway Analysis

We carried out pathway analysis using DIANA-mirpath webservice http://diana.imis.athena-innovation.gr/DianaTools (Vlachos et al. 2015), which employs DIANA-Tarbase algorithm to predict the miRNA targets. Pathway analysis was carried out for ten validated miRNAs, and to determine the specific targeted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for ten miRNAs, pathway union feature was selected in the mirpath tool.
Target Prediction

The potential predicted gene targets of the validated differentially expressed miRNA were detected using open-access web server Diana microT-CDS (Paraskevopoulou et al. 2013; Reczko et al. 2012). Except for TGF-β1, other gene targets were co-predicted either with miRWalk 3.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk3/) an atlas of predicted and validated miRNA-target associations and miRDB v4.0 (http://www.microrna.org/) online database for miRNA target prediction and functional annotations (Dweep et al. 2011; Dweep and Gretz 2015).

Enrichment Analysis

To identify the biological functions of the dysregulated miRNAs, BiNGO v3.0.3, (Maere et al. 2005) Cytoscape plugin software was used. GO enrichment analysis was carried out by the hypergeometric test using Benjamin-Hochberg false discovery rate (FDR) correction $P$ value less than 0.05. The overall design of the present study is shown in the flow chart in Fig. 1.

Sample Collection and Transforming Growth Factor-Beta (TGF-β) ELISA Assay

Venous blood (5 ml) was drawn into plain BD Vacutainers (Becton Dickinson, India) tubes and allowed to stand for 45 min at room temperature. Samples were then centrifuged at 2800 rpm for 15 min at room temperature. Serum TGF-β level was determined in all subjects by enzyme-linked immunosorbent assay (ELISA) (Ray Biotech, USA) as per the manufacturer’s protocol.

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism v.5.0 (Graph Pad Software) and SPSS version 22.0 (IBM Corporation, NY, USA). Fisher’s exact test was used to compare general characteristics between controls and cases. Because the data did not meet normal distribution (Shapiro–Wilk test); we used non-parametric Mann–Whitney $U$-tests and Spearman correlation to carry out the analysis (Supplementary Table 3 in ESM_2). Mann–Whitney $U$-tests were used to calculate the statistical significance of various miRNA/mRNA and TGF-β levels. Spearman correlation coefficient analysis was used to assess the correlation between tissue miRNA and mRNA expression. The level for statistical significance was set at a $p$-value less than 0.05.

Results

Study Subjects and Patient Characteristics

Surgically resected aneurysm tissue from 29 aSAH patients (mean age, 52.38 ± 2.29 years) and 20 intercostal arteries which were harvested in healthy adult patients undergoing surgery for the repair of brachial plexus injury (mean age, 48.30 ± 4.07 years) were included for the analysis. The baseline characteristics of aSAH patients and controls are shown in Table 1. No significant difference was noted between cases and controls in terms of the distribution of age, gender and alcohol drinking status. As expected, hypertension was more prevalent in aSAH patients ($p = 0.037$, Fisher’s exact test). The clinical features and the characteristics of the aneurysms are detailed in Tables 2 and 3.

Screening of Differentially Expressed miRNA in Aneurysm and Control Tissues

The miRNA microarray analysis from aneurysm vessel walls and control tissues (RNA from 8 aneurysm walls and 8 control tissues were pooled, respectively) revealed 70 differentially expressed miRNA in the aneurysm vessels compared to control tissues (absolute value of log2 fold change greater or lesser than 2). Among them, 67 miRNAs were down-regulated, and 3 were up-regulated (Supplementary Table 4 in ESM_2). As shown in Fig. 2a, two-way hierarchical clustering analysis indicated a well-defined configuration.
of miRNA expression in aSAH patients and controls. Principal component analysis (PCA) showed a divergent pattern of miRNA expression between cases and controls. The illustrations of a traditional and a matrix PCA plot are represented in Supplementary Fig. 1 in ESM_1.

Differentially Expressed miRNA in Aneurysm and Control Tissue Samples Analyzed by RT-qPCR

We selected 10 differentially expressed tissue miRNAs (miR-24-3p, miR-26b-5p, miR-27b-3p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-193a-3p, miR-199a-5p, miR-365a-3p/365b-3p, and miR-497-5p) based on the magnitude of fold change between duplicates. The expressions of these selected miRNAs were validated individually on a new set of 21 aneurysm tissue samples and 12 controls including the individual biological samples that were used in the microarray study (8 aneurysm tissue samples and 8 control tissues). The fold-change of these 10 tissue miRNA as determined by microarray is shown in Fig. 2b. The relative expression levels of the 10 tissue miRNAs by qRT-PCR were significantly down-regulated ($p < 0.05$, Mann–Whitney U test) in aneurysm tissues ($n = 29$) compared to control tissues ($n = 20$) (Fig. 2c).

Association Between miRNA Expression in Aneurysm Tissues and Clinical Characteristics of aSAH Patients

The expression of aneurysm tissue miRNAs was correlated with clinical characteristics of aSAH patients, including WFNS grade, vasospasm, and clinical outcome. Mann–Whitney $U$ test show that the expression of miR-125b-5p ($U = 32.5$, $p = 0.016$), miR-143-3p ($U = 36.5$, $p = 0.025$) and miR-199a-5p ($U = 30.5$, $p = 0.027$) was significantly decreased in aneurysm tissues of patients with WFNS grade 3 & 4 (Mdn = 0.300; Mdn = 0.044; Mdn = 0.394) compared to those with WFNS grade 1 & 2 (Mdn = 0.659; Mdn = 0.084; Mdn = 1.039) (Fig. 3a). Similarly, expression of miR-125b-5p and miR-143-3p was significantly decreased ($U = 23$, $p = 0.037$ and $U = 26.5$, $p = 0.043$ respectively) in patients with vasospasm (Mdn = 0.339, Mdn = 0.045) compared to patients without vasospasm (Mdn = 0.659, Mdn = 0.096) (Fig. 3b). Although the expression of these 10 differentially expressed tissue miRNAs (miR-24-3p, miR-26b-5p, miR-27b-3p, miR-125b-5p, miR-143-3p, miR-145-5p, miR-193a-3p, miR-199a-5p, miR-365a-3p/365b-3p, and miR-497-5p) was associated with the clinical outcome, it was not statistically significant (Fig. 3c).

Identification of Putative Target Genes of miRNA and Their Functional Analysis

The genes targeted by the top 10 downregulated miRNAs were predicted using the online tool, miRWalk 3.0 databases. The miRNA-targets pairs were selected based on experimentally confirmed miRNA-target interaction. As a
result, 4531 genes were predicted as possible targets of the dysregulated miRNAs. Since several miRNAs target multiple genes, the total unique genes were 3822. The distributions of genes targeted by each candidate miRNA are presented in a Venn diagram (Fig. 4a). KEGG pathway analysis for all top 10 downregulated miRNAs and the number of involved genes and their statistical significance are shown in Fig. 4b and Supplementary Table 5 in ESM_2. Using the DIANA-miRPath, we determined the biological functions of the differentially expressed top 10 downregulated miRNAs. The networks of these miRNAs and the predicted pathways were constructed using Cytoscape software. Major signaling pathways of these miRNA, including TGF-β, MAPK, NF-kB, focal adhesion, and calcium signaling pathway, were identified as potential pathways (Fig. 4C). The biological significance of the top 10 downregulated miRNAs was analyzed using the GO terms at three levels to gain insight into the cellular components, biological processes, and molecular function associated with the pathogenesis of aneurysm formation and rupture (Fig. 5). We found that the top 10
Fig. 4  Putative target genes of differentially expressed tissue miRNA. 
(a) Venn diagram illustrating the number of genes targeted by each of the tissue miRNAs. (b) Heat map of the KEGG pathway. 10 tissue miRNAs involved in intracranial aneurysm signaling pathways. (c) The major signaling pathway enrichment analysis of the 10 tissue miRNA (The shade of the red color represents the most prominent pathways involved in aneurysm pathogenesis).

Fig. 5  Gene ontology enrichment analysis based on validated 10 tissue miRNA-target genes. The bar plot shows the enrichment scores ($P$ value) of the significant enrichment GO terms. Target genes are associated with cellular components, biological processes and molecular function.
downregulated miRNAs were significantly enriched with GO terms linked to signal transduction, cell proliferation, and differentiation, cytoskeleton and mitochondrial organization, cell–cell signaling, metabolic process and response to stress. These processes are directly or indirectly related to aneurysm pathogenesis.

RT-qPCR Validation of mRNA Targets and Their Correlation with Targeting miRNAs (TGF-β and MAPK Signaling Pathway)

Based on the bioinformatic target prediction platform, we identified TGF-β and MAPK pathway components, TGF-β1, TGF-β2, TGF-β3, SMAD2, SMAD4, MAPK1, and MAPK3, as putative predictive targets of miR-26b, miR-199a, miR-365, and miR-497.

Role of miRNAs in the TGF-β Signaling Pathway

We found that the downregulated miR-26b, miR-199a, miR-365 targeted TGF-β1, TGF-β2, TGF-β3 and miR-26b and miR-497 were predicted to act with 3′UTR of SMAD2 and SMAD4 (Figs. 6a and 7a). The mRNA levels of TGF-β1, TGF-β2, SMAD2, and SMAD4 (U = 72, p = 0.0001; U = 155, p = 0.0053, U = 123, p = 0.0002; U = 119, p = 0.0053) were significantly higher in aneurysm tissues (Mdn = 0.956; Mdn = 3.105; Mdn = 1.665; Mdn = 0.756) compared to control tissues (Mdn = 0.123; Mdn = 1.333; Mdn = 0.212; Mdn = 0.196) (Figs. 6b and 7b). Although, mRNA levels of TGF-β3 were higher in aneurysm tissues compared to controls, it was not statistically significant (U = 239; p = 0.304) Serum TGF-β levels (U = 1204, p = 0.0002) measured by ELISA were significantly elevated in aSAH patients (Mdn = 4.545) compared to controls (Mdn = 4.183) (Fig. 6d). Next, we studied the relationship between the miRNA expression and the levels of their target genes. As shown in Figs. 6c and 7c, the three miRNA (miR-26b, miR-199a, miR-497) exhibited significantly inverse correlation with TGF-β1, SMAD2 TGF-β2, and SMAD4 (r = −0.318, p = 0.045; r = −0.330, p = 0.040; r = −0.338, p = 0.023; r = −0.372, p = 0.036 respectively, Spearman correlation coefficient).

MiRNAs in MAPK/ERK Signaling Pathway

The downregulated miR-365 and miR-497 were predicted to act with the 3′UTR of MAPK1 and MAPK3 (Fig. 8a). Although mRNA levels of MAPK1 were higher in aneurysm tissues compared to controls, it was not statistically significant (U = 196; p = 0.076) (Fig. 8b). However, MAPK3 expression was significantly elevated (U = 104, p = 0.007) in aneurysm tissues (Mdn = 2.076) compared to controls (Mdn = 2.076). As shown in Fig. 8c, the two miRNA (miR-365, and miR-497) exhibited a significant inverse correlation with MAPK1, and MAPK3 (r = −0.362, p = 0.037; P < 0.05). c Spearman’s correlation analysis of the relative expression levels of miR-26b, miR-199a and miR-365 and the relative expression levels of TGF-β1, TGF-β2 and TGF-β3 mRNA. d TGF-β concentration was measured in the serum of aSAH patients (n = 70) and controls (n = 60) by ELISA.

**Fig. 6** MiRNA interaction with TGF-β a Schematic representation of miR-26b, miR-199a and miR-365 binding sequence in the 3′-UTR of TGF-β1, TGF-β2 and TGF-β3 mRNA. b qRT-PCR analysis of TGF-β1, TGF-β2 and TGF-β3 mRNA expression in control (n = 20) and aneurysm tissues (n = 29). Data are represented as mean ± SD (P < 0.05). e Spearman’s correlation analysis of the relative expression levels of miR-26b, miR-199a and miR-365 and the relative expression levels of TGF-β1, TGF-β2 and TGF-β3 mRNA. d TGF-β concentration was measured in the serum of aSAH patients (n = 70) and controls (n = 60) by ELISA.
Fig. 7 Mir-497 and miR-26b targets SMAD2 and SMAD4 genes in the TGF-β signaling pathway. a Schematic representation of mir-497 and miR-26b interaction with 3′ UTR of SMAD2 and SMAD4 mRNA. b qRT-PCR showing the mRNA expression of SMAD2 and SMAD4 expression in control (n = 20) and aneurysm tissues (n = 29). Data are represented as mean ± SD (P < 0.05). c Spearman’s correlation analysis of mir-497 and miR-26b and its targets presented as scatter plots.

Fig. 8 MiRNA interaction with the MAPK signaling pathway. a Schematic representation of miR-365 and miR-497 interaction with 3′ UTR of MAPK1 and MAPK3 mRNA. b qRT-PCR showing the mRNA expression of MAPK1 and MAPK3 expression in control (n = 20) and aneurysm tissues (n = 29). Data are represented as mean ± SD (P < 0.05). c Spearman’s correlation analysis of miR-365 and miR-497 and its targets presented as scatter plots.
Dysregulated miRNAs have been associated with the etiopathogenesis of many cerebrovascular disorders, and altered plasma miRNA expression has been previously reported in aSAH (Jamaluddin et al. 2011). In our study, using miRNA microarray analysis we identified 70 miRNAs with significantly altered expression (absolute value of the log fold-change (base 2) is larger or less than 2) in aneurysm tissues compared to control tissues: 67 were down-regulated, and 3 were up-regulated. The top 10 dysregulated miRNAs were further validated individually using qPCR, and these miRNAs were observed to be significantly downregulated in aneurysm tissues.

Recently, limited miRNA expression studies have identified several differentially expressed miRNA in aneurysm tissue (Bekelis et al. 2016; Liu et al. 2014). However, their results are not readily comparable, which may be due to the use of different analytical methods (different microarray-based methodologies, qPCR and sequencing), validation strategies (independent testing vs no validation vs validation in the same cohort) and control tissues. Meanwhile, we further explored the association of the differentially expressed miRNA with the clinical characteristics of patients. Expression levels of 2 miRNAs (miR-125b-5p and miR-143-3p) were considerably lower in patients with vasospasm compared to patients without vasospasm. Moreover, expression of miR-125b-5p, miR-143-3p and miR-199a-5p was significantly down-regulated in patients with WFNS grades 3 & 4 compared to those with WFNS grade 1 & 2. Previous studies report that miR-143/miR-145 is highly expressed in vascular smooth muscle cells, endothelial cells, and inflammatory cells (Cheng et al. 2009). Also, it has been suggested that miR-143/mir-145 are critical modulators of vascular smooth cell phenotype in response to shear stress in atherosclerosis and hypertension (Santovito et al. 2013). In addition, a prior study by Bekelis et al. in patients with unruptured aneurysms reported that miR-143 is significantly downregulated and contributed to the modulation of vascular smooth muscle cell phenotype (Bekelis et al. 2016). A recent study showed that miR-125b-5p regulates both the innate immune response and the inflammatory process by directly targeting the expression of a gene encoding 5-lipoxygenase enzyme involved in the biosynthesis of leukotrienes (Busch et al. 2015). Furthermore, miR-125b is reported to be associated with cell proliferation, apoptosis, and vascular smooth cell phenotyping in aneurysms (Liu et al. 2014; Robinson and Baker 2012). Thus, expression of miR-125b-5p and miR-143-3p could be related to disease progression or severity, and could perhaps predict clinical outcome.

It is well known that in humans, conserved miRNA preferentially targets many sets of mRNA, which play vital roles in multiple biological pathways (Cortez et al. 2011). However, the contribution of gene networks targeted by the deregulated miRNAs in aneurysm biology is not known. Although few studies explicitly link miRNA to its downstream target gene, no study has experimentally validated the gene targets of miRNAs in aneurysm tissue. To address this, we used the interaction of the experimentally verified miRNA with the gene information module to find the genes targeted by the miRNAs and found that these miRNAs targeted the expression of 3822 unique genes in aneurysm tissue. KEGG pathway analysis indicated that the target genes of the dysregulated tissue miRNA were exclusively associated with several signaling pathways, including, TGF-β, MAPK, and NF-kB signaling pathway underlying its importance in aneurysm biology.

In recent years, the TGF-β signaling pathway has gained special attention since TGF-β is a multifaceted cytokine that regulates a diverse range of cellular activities (Morikawa et al. 2016; Moustakas et al. 2002). Several studies report that numerous miRNAs can induce TGF-β, and it is a chief domain that activates key components of the downstream cell signaling cascade. Various reports suggest that TGF-β is essential for maintaining vascular integrity and function. In rats, middle cerebral artery occlusion resulted in elevated TGF-β expression (Vincze et al. 2010). Experimental evidence in mice reported that TGF-β signaling amplifies with an increase in age and signals to innate immune cells and astrocytes after stroke (Doyle et al. 2010). The relationship between TGF-β and IA is still poorly understood. Mutation in the genes encoding ENG/endoglin and TGFBR3/betaglycan transmembrane proteins that modulate TGF-β predispose to aneurysm pathogenesis (Santiago-Sim et al. 2009). In our present study, we found that the expression of miR-26b and miR-199a markedly decreased and the expression of TGF-β1 and TGF-β2 in aneurysm tissue increased compared to controls. In addition, 3’UTR of SMAD2 and SMAD4 mediator of TGF-β signal transduction was targeted by miR-497 and miR-26b. Expression of miR-497 and miR-26b was downregulated and SMAD2 and SMAD4 levels were overexpressed in aneurysm tissue. These findings suggest that abnormally expressed miR-26b, miR-199a and miR-497 may play a role in aneurysm biology through TGF-β signaling mediated by SMAD2 and SMAD4 transcription factors.

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases, and the two MAPKs: MAPK1/ERK2 and MAPK3/ERK1, play central roles in the MAPK/ERK signaling. Typically, they control various transcription factors that govern the transcription of an array of genes involved in endothelial cell proliferation, cytoskeletal, and
vascular remodeling by phosphorylating protein kinases (Bogatcheva et al. 2003; Plotnikov et al. 2011). Also, MAPK cascade is activated by a wide range of extracellular stimuli such as growth factors, cytokines, and also in response to cellular stress (Yoon and Seger 2006). In experimental animals, rapid activation of ERK was seen in balloon-injured arteries, hypertensive vascular tissue (Kim et al. 1998). In addition, ERK was activated in rat aortic vascular smooth muscle cells in vitro in response to both cyclic strain and shear stress (Hu et al. 1998). Interestingly, ERK activation in cultured endothelial cells was higher in response to shear stress compared to cyclic strain (Azuma et al. 2000). Furthermore, the involvement of MAPKs in the regulation of miRNAs expression has also been reported (Hong et al. 2013). However, the role of miRNA and MAPKs in the pathobiology of IAs is unclear. In this study, we examined the expression of miR-365 and miR-497, targeting MAPK1 and MAPK3, respectively, in aneurysm tissues. Our study revealed that the expression of miR-365a and miR-497 was decreased, and MAPK1 and MAPK3 increased in aneurysm tissues compared to controls suggesting a role for miRNA in regulating the stress-activated kinase, ERK, in the aneurysm wall. Our results are supported by a study by Maddahi et al., which showed that the ERK1/2 pathway was activated in IA tissues post-SAH (Maddahi et al. 2012).

We have systematically analyzed the biological functions and downstream signaling pathways associated with target genes of dysregulated tissue miRNAs (Fig. 9). These data suggest that miRNA could play a significant role in the dysfunction and remodeling of vascular endothelial and smooth muscle cells by influencing inflammatory immune processes via the downstream regulation of genes, which could, in turn, contribute to the pathophysiology of aneurysm rupture and aSAH.

Our study has a few limitations. Since we included a relatively small number of patients in the validation cohort, we failed to define a significant association between miRNA expression and clinical outcome. In addition, as normal cerebral arteries are difficult to harvest for use as control tissues, we have obtained control tissues from patients without any known brain or vascular pathologies. Intercostal arteries harvested during brachial plexus injury repair which was carried out in healthy adults after vehicular accidents were used as controls, and therefore, region-specific differences in miRNA expression in the vascular tissues, cannot be ruled out. Also, target mRNA of tissue miRNAs was validated based on functional analysis and we failed to validate these putative miRNA targets in vivo. Further, TGF-β protein levels were measured in serum only, because the residual aneurysmal wall obtained after the surgery was very small, and consequently, the observed changes in serum TGF-β protein levels may not imply a causal relationship with the IA tissue content. An additional limitation is that miRNA microarray profiling can only measure miRNAs that are included in the array and cannot discover new miRNAs.

To conclude, this study identified several differentially expressed miRNAs in ruptured IA tissue through microarray analysis. Bioinformatic analysis showed that miR-26b, miR-199a, miR-497 and miR-365 which were significantly decreased, modulate genes involved in TGF-β and MAPK signaling, which could potentially influence inflammatory processes, extracellular matrix and vascular smooth muscle cell degradation and apoptosis, and ultimately cause vessel wall damage and rupture. Further functional validation of the target genes of the dysregulated miRNA are necessary for deciphering their exact role in the progression and rupture of IAs.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10571-021-01121-3.

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Authors Contributions CR, IDB, BDL, SD designed the study. SM and CR collated the data, carried out data analyses and produced the initial draft of the manuscript. KSR provided technical assistance for analysis.
and interpretation of data. All authors discussed the results, provided critical suggestions, and approved the manuscript.

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**Data Availability** The datasets generated during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no competing interests.

**Ethical Approval** This study was approved by the Ethical Committee of National Institute of Mental Health and Neuro Sciences (No.NIMH/DO/ETHICS SUB-COMMITTEE 11th MEETING/2015).

**Informed Consent** Written informed consent was obtained from all subjects or their legal guardians or to participate in the study.

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