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.

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 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 center. 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 intercostomusculocutaneous 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), an RNA stabilization solution, and stored at -80°C till analysis.

Table 1
General characteristics of controls and patients with aSAH

Parameters	Controls	Patients	P value
	n = 20	n = 29	
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.			

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 2004). Further, based on the volume of blood observed on the brain CT scan, 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 1.8-2.1 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 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 on samples pooled from each group - 8 aneurysm walls and 8 control tissues (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 image analysis was performed by 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 NCBIs 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^{TM} 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 (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 100ng 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 Ct values were normalized to miR-150 for miRNA and GAPDH for mRNA. Relative miRNA/mRNA expression was calculated by the $2^{\wedge\text{-}\Delta\Delta Ct}$ method. Data were represented as fold change.

Functional analysis of targeted miRNAs

Pathway analysis

We carried out pathway analysis using DIANA-mirpath web server 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 mirpath tool.

Target prediction

The potential 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), which identified those targets that were co-predicted either with miRWalk3.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) 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 *w*as 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 45mins at room temperature. Samples were then centrifuged at 2800rpm for 15mins 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 arterial tissues from the calf obtained during removal of the sural nerve during peripheral nerve surgery in patients (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 Table 2 and Table 3.

Table 2
Clinical characteristics of aSAH patients

Clinical details of aSAH patients	**N = 29 (%)**
Headache	27 (93.10)
Vomiting	23 (79.31)
Altered sensorium	11 (37.93)
Focal deficits	3 (10.34)
Seizures	4 (13.79)
WFNS Grade	
Grade I	11 (37.93)
Grade II	5 (17.24)
Grade III	6 (20.68)
Grade IV	7 (24.13)
Fisher scale (CT scan)	
Fisher grade 1	—
Fisher grade 2	2 (6.89)
Fisher grade 3	21(72.41)
Fisher grade 4	6 (20.68)

Table 3
Characteristics of aneurysm in aSAH patients

Characteristics of IA	N = 29 (%)
Site of aneurysm	
Anterior communicating artery	14 (48.27)
Middle cerebral artery	3 (10.34)
Internal carotid artery	2 (6.89)
Posterior communicating artery	1 (3.44)
Distal anterior cerebral artery	4 (13.79)
Posterior inferior cerebellar artery	1 (3.44)
Multiple	4 (13.79)
Size of aneurysm	
Small(< 5mm)	22 (75.86)
Large (5-7mm)	5 (17.24)
Giant (> 10mm)	2 (6.89)
No. of aneurysm	
Single	25(86.20)
Multiple	4(13.79)
Outcome	
Dead	2 (6.89)
Vegetative state	1 (3.44)
Severely disabled	1 (3.44)
Moderately disabled	6 (20.68)
Good recovery	19(65.51)

Screening of differentially expressed miRNA in aneurysm and control tissues

The miRNA microarray analysis from aneurysm vessel walls (n = 8) and intercostal arteries (n = 8) revealed 70 differentially expressed miRNA in the aneurysm vessels compared to control tissues (Fold

change </>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.

Validation of differentially expressed miRNA in aneurysm and control tissues

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 microarray study (8 aneurysm tissue samples and 8 control tissues). The relative expression levels of the 10 tissue miRNAs were significantly down-regulated ($p < 0.05$, Mann-Whitney U test) in aneurysm tissues ($n = 29$) compared to control tissues ($n = 20$) (Supplementary Table 5 in ESM_2). The fold-change of these10 tissue miRNA is shown in Fig. 2b.

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 vasospasm ($U = 23$, $p = 0.037$ 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 miRNA 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, miRWalk2.0 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 miRNAs 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 6 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 downregulated miRNAs were significantly enriched with GO terms linked to signal transduction, cell proliferation, and differentiation, cytoskeleton and mitochondrial organization, cell-cell signalling, metabolic process and response to stress. These processes are directly or indirectly related to aneurysm pathogenesis.

Validation of putative target genes of TGF-β and MAPK signaling pathway by miRNAs

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

Role of miRNAs in 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* (Fig. 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) (Fig. 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 Fig. 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 *MAPK*3 (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 significantly inverse correlation with *MAPK1*, and *MAPK3 (*r=-0.362, p = 0.037; r=-0.378, P = 0.013; respectively, Spearman correlation coefficient).

Discussion

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 (log fold-change greater 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. 2016a; Liu et al. 2014a). 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. Mean while, 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. 2016b). 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. 2014b; 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 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 which 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 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 over expressed 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 few limitations. Since we included a relatively small number of patients in the validation cohort, we failed to define significant association between miRNA expression and clinical outcome. Further, target mRNA of tissue miRNAs was validated based on functional analysis and we failed to validate these putative miRNA targets in-vivo. 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 intracranial aneurysmal tissue through microarray analysis. Bioinformatic analysis showed that miR-26b, miR-199a, miR-497 and miR-365 which were significantly decreased, modulates 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 degradation and rupture. Further functional validations of the target genes of the dysregulated miRNA are necessary for deciphering their exact role in the progression and rupture of IAs.

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

Declarations

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Authors Contributions:

Christopher R, Indira Devi B, Bhat DI, Shukla D designed the study. Supriya M and Christopher R collated the data, carried out data analyses and produced the initial draft of the manuscript. All authors discussed the results, provided critical suggestions, and approved the manuscript.

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Compliance with Ethical Standards

Disclosures:

The authors declare that they have no competing interests.

Data Availability

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

Ethical approval and informed consent:

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). Written informed consent was

obtained from all subjects or their legal guardians or to participate in the study.

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Figures

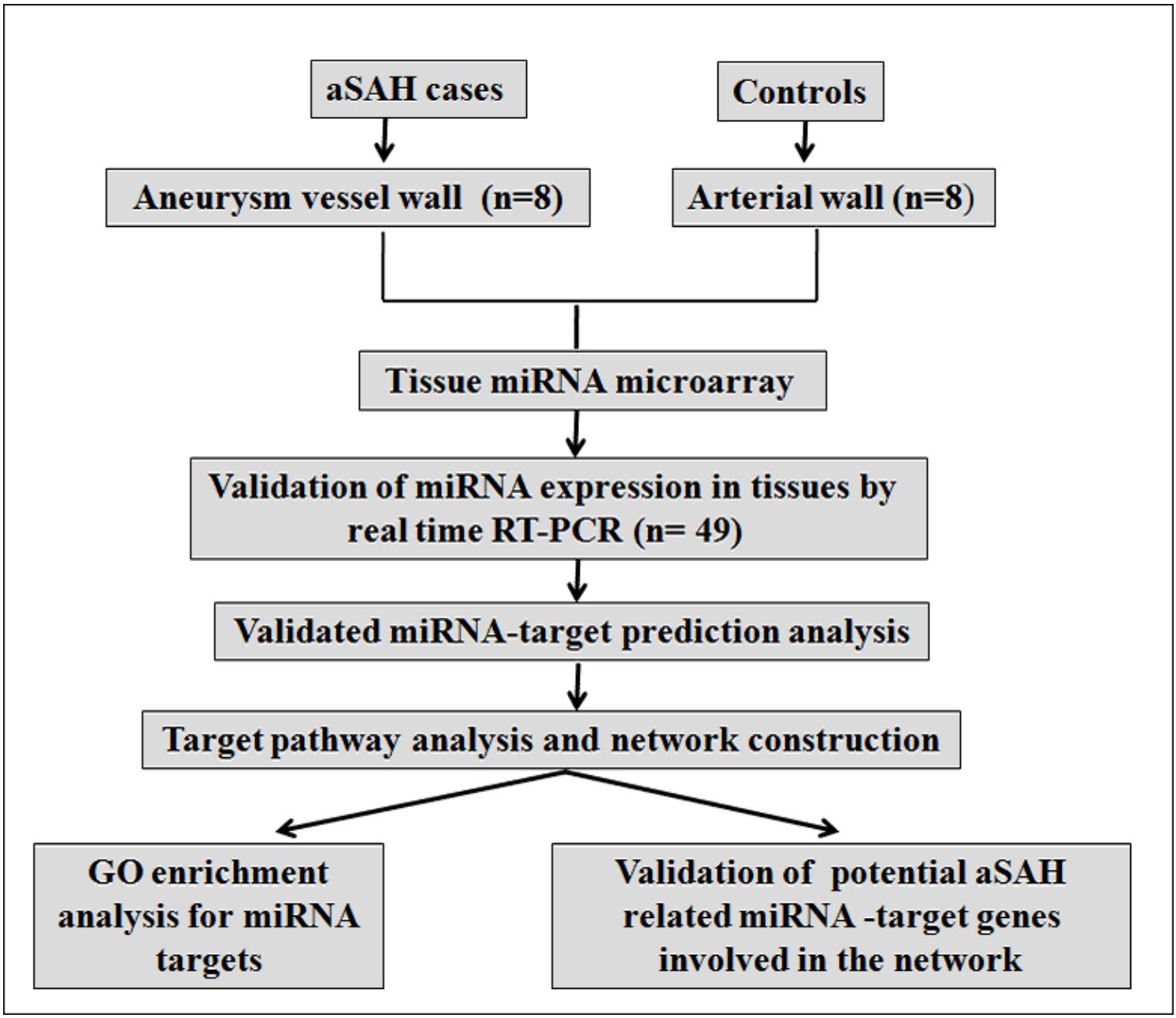

Figure 1

Overview of the tissue microRNA expression analysis in the present study. GO: gene ontology, aSAH: aneurysmal subarachnoid hemorrhage, miRNA: microRNA, qRT-PCR: quantitative real-time polymerase chain reaction.

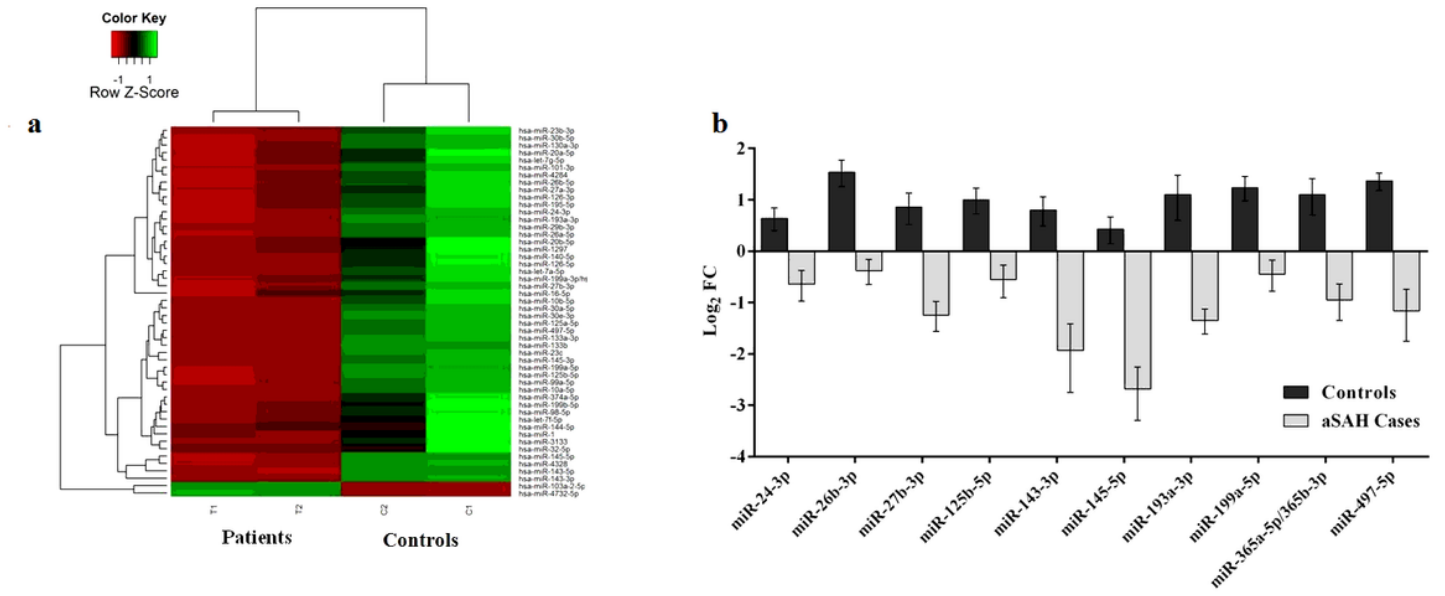

Figure 2

Differentially expressed tissue miRNA in intracranial aneurysmal tissues and control tissues. a: The heat map diagram shows the results of the two-way hierarchical clustering of miRNAs and samples. Each row represents one miRNA, and each column represents sample. The miRNA clustering tree is shown on the left. The color scale at the bottom illustrates the relative expression level of a miRNA across all samples: red represents an expression level above mean, green represents expression lower than the mean. b: The FC of miRNA expression in aneurysm tissues compared with control tissues. The log2 (FC) between cases and controls is displayed in the Y-axis. Columns: tissue miRNAs. Baseline: tissue miRNAs from controls.

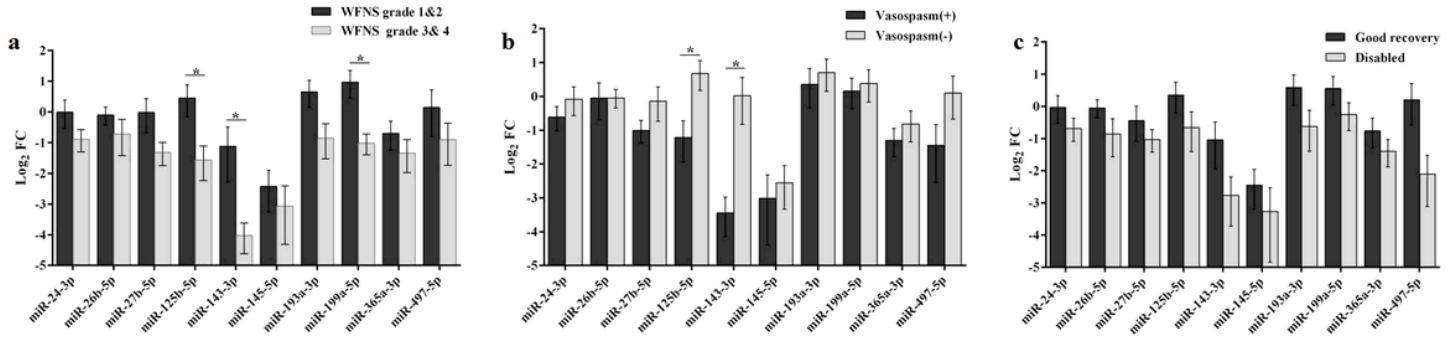

Figure 3

Distinct pattern of tissue miRNA expression. a: Tissue miRNA expression pattern among aSAH patients with different WFNS grades (I to IV). b: Tissue miRNA expression pattern of aSAH patients, with and without vasospasm. c: Tissue miRNA expression pattern with the clinical outcome of aSAH patients.

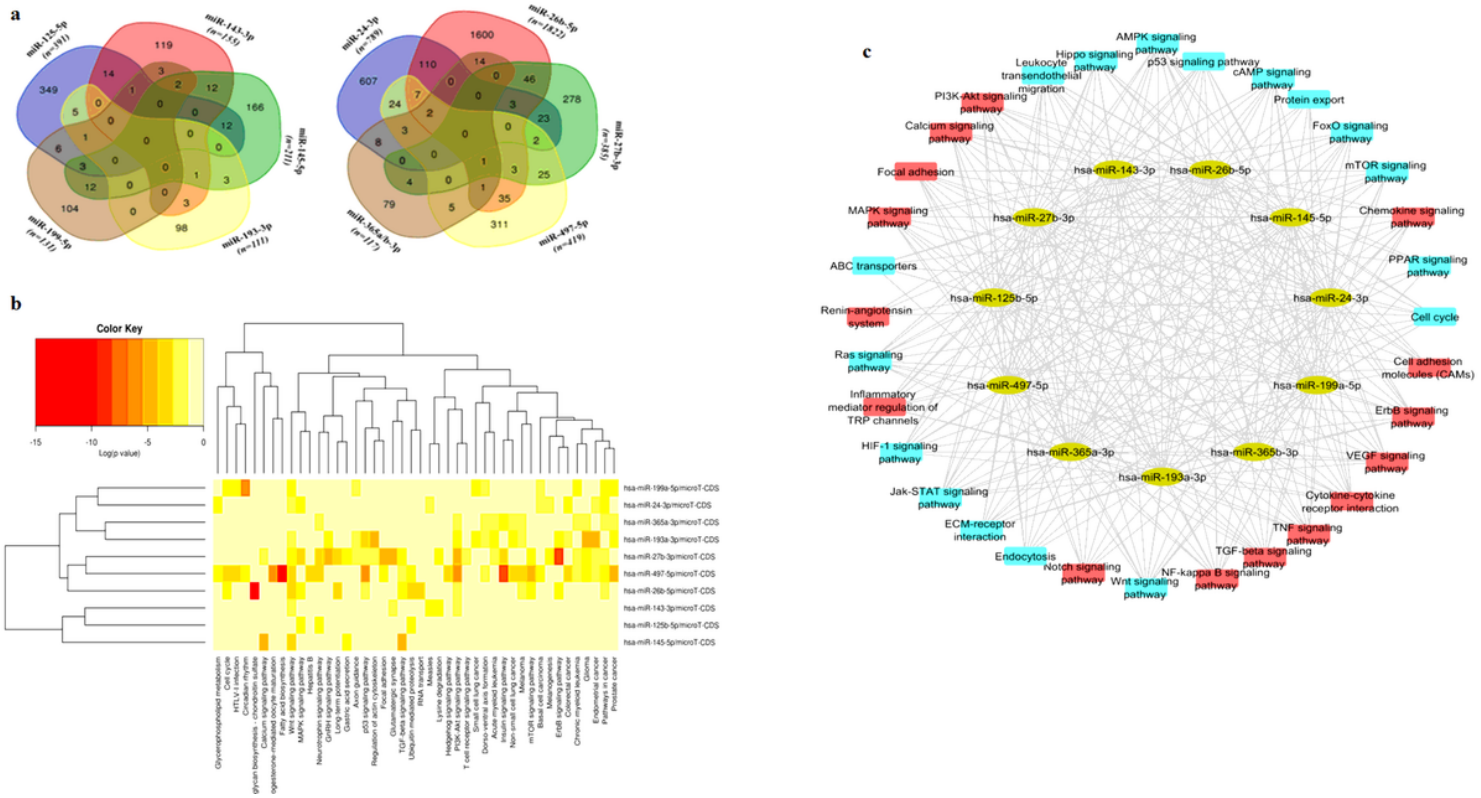

Figure 4

Putative target genes of differentially expressed tissue miRNA. a: Venn diagram illustrating 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 red color represents most prominent pathways involved in aneurysm pathogenesis).

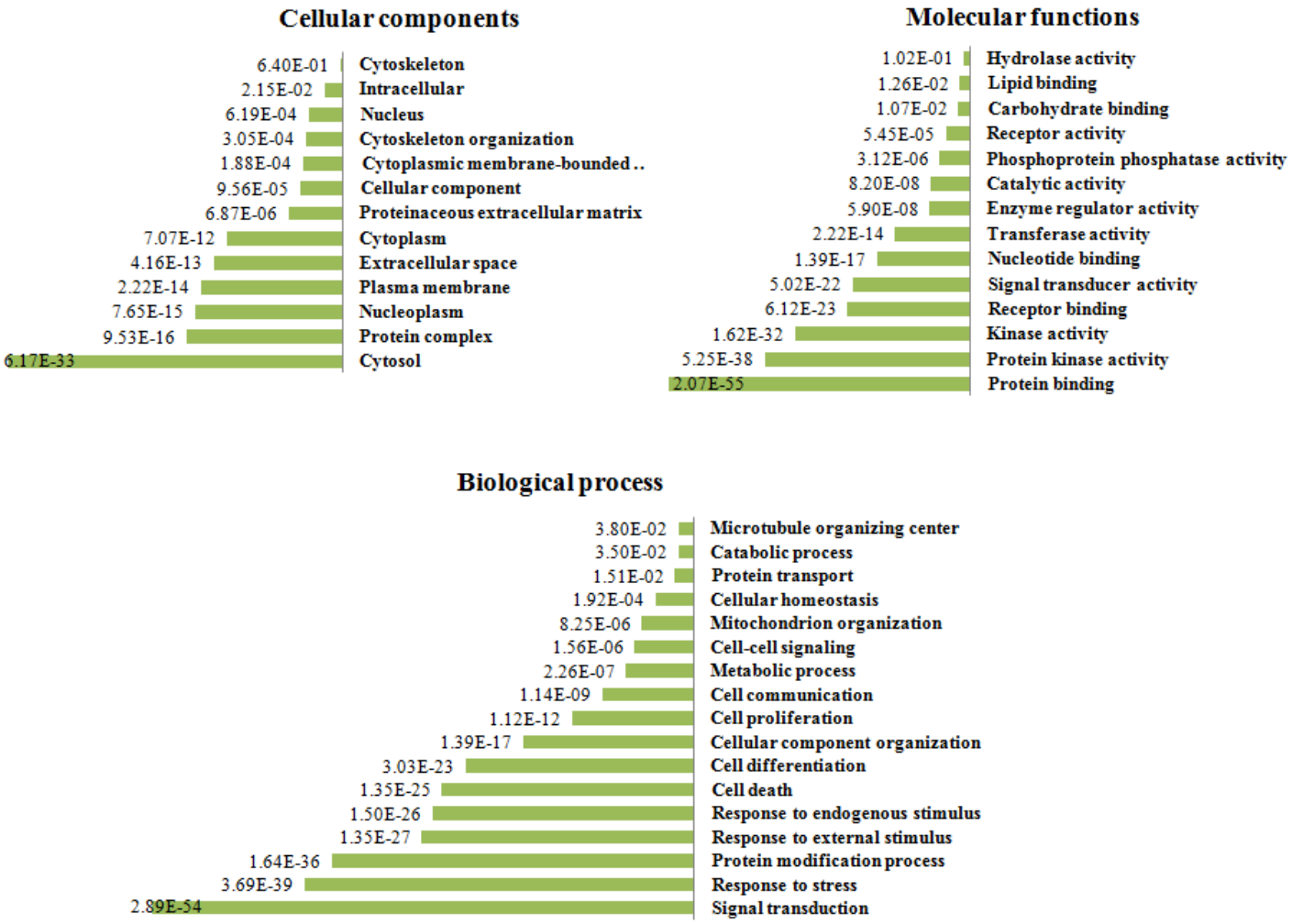

Figure 5

Gene ontology enrichment analysis based on validated 10 tissue miRNA-target genes. Target genes are associated with cellular components, biological processes and molecular function.

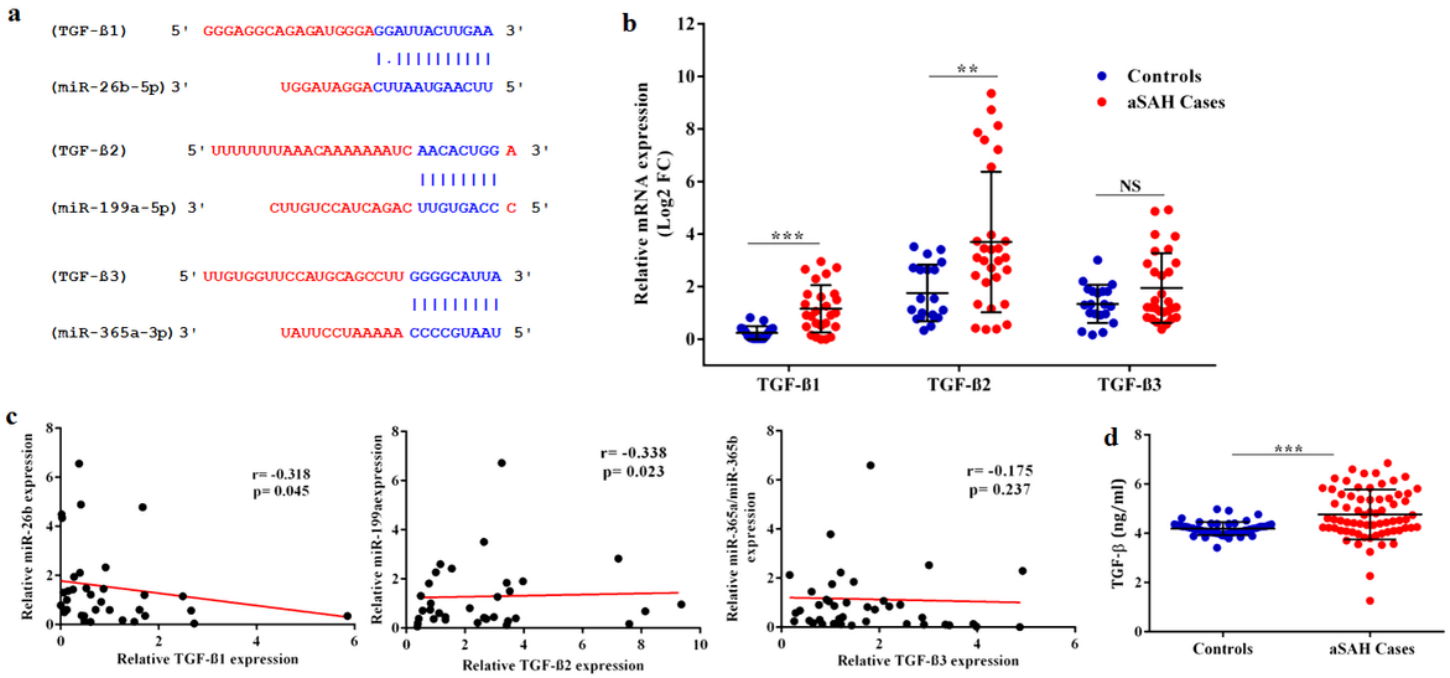

Figure 6

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 and aneurysm tissues. Data are represented as mean ± SD ($P < 0.05$). 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 and controls by ELISA

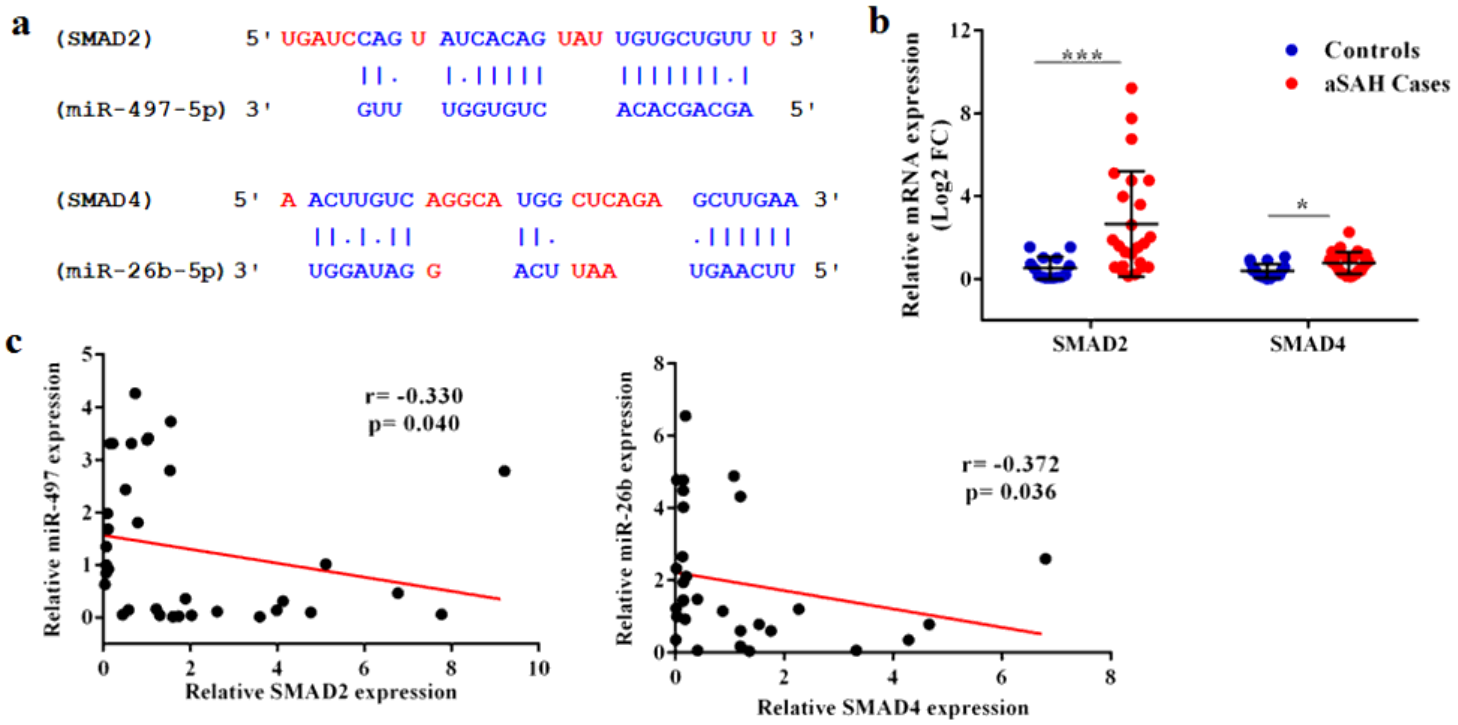

Figure 7

Mir- 497 and miR-26b targets SMAD2 and SMAD4 genes in 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 and aneurysm tissues. Data are represented as mean ± SD ($P < 0.05$). c: Spearman's correlation analysis of mir-497and miR-26b and its targets presented as scatter plots.

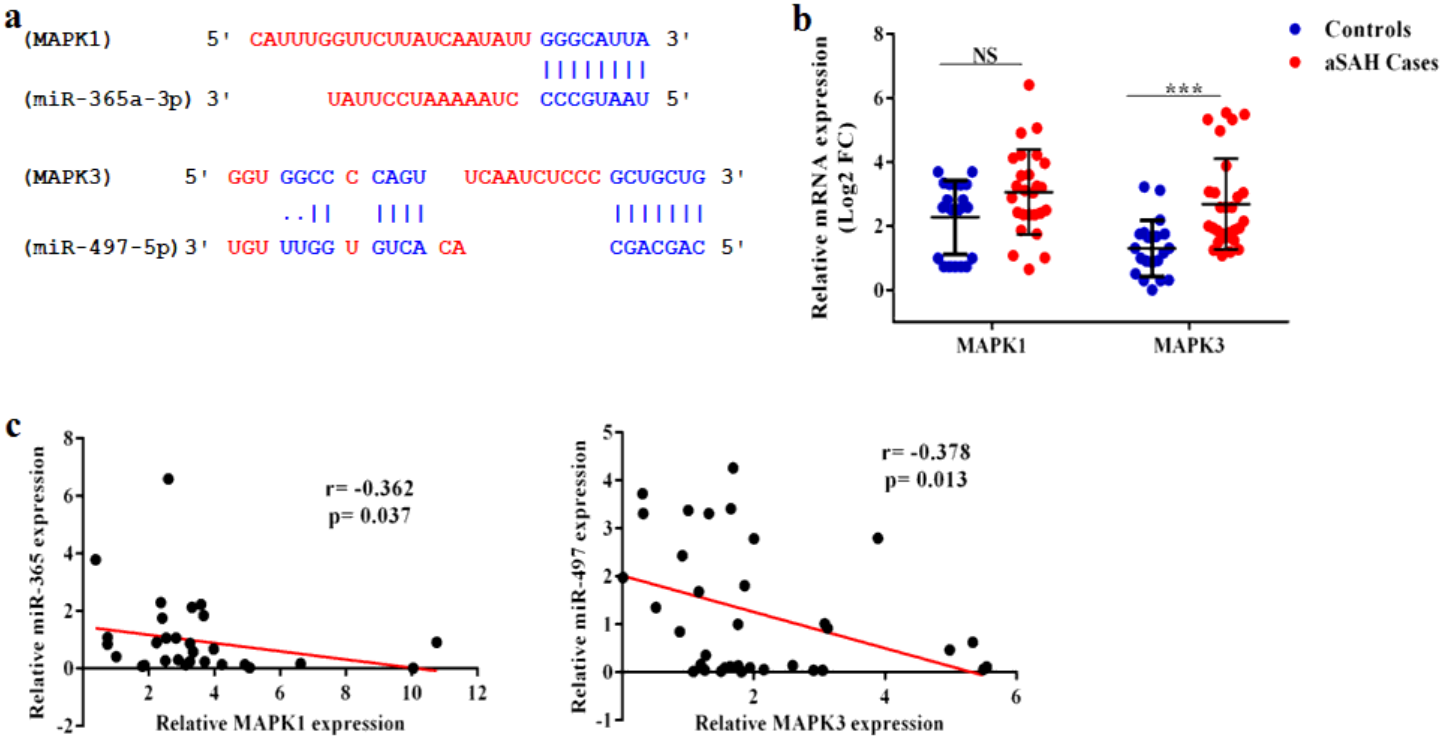

Figure 8

MiRNA interaction with 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 and aneurysm tissues. Data are represented as mean ± SD ($P < 0.05$).). c: Spearman's correlation analysis of of miR-365 and miR-497 and its targets presented as scatter plots.

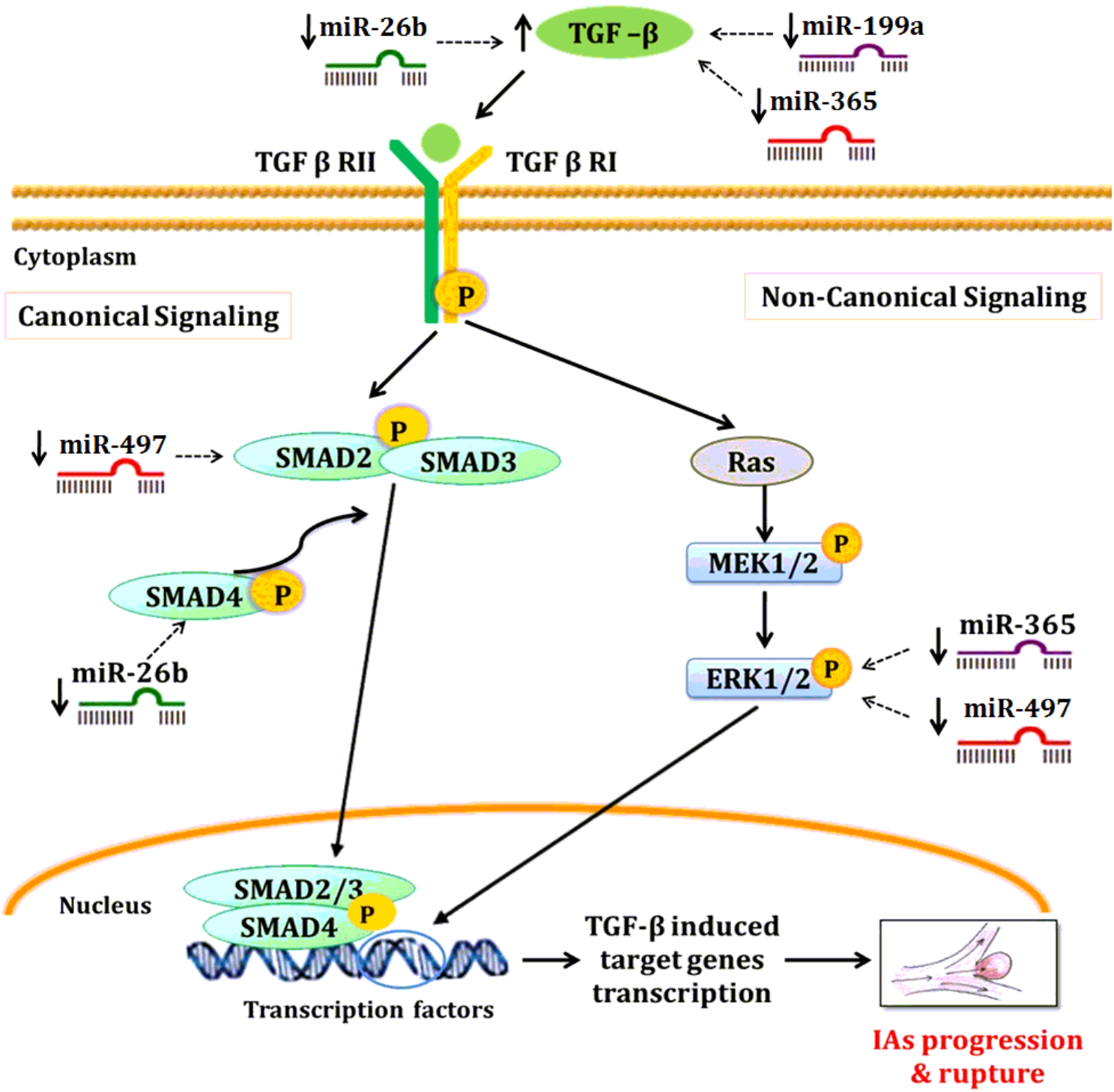

Figure 9

Possible mechanism of involvement of dysregulated network of miRNAs in intracranial aneurysm pathogenesis. Dysregulated miRNAs lead to the activation of the TGF-β signaling pathway, which triggers abnormal inflammatory processes, extracellular matrix and vascular smooth muscle cell degradation and apoptosis, which could ultimately cause vessel wall degradation and rupture.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ESM1.pdf
- ESM2.pdf