miRNA expression profiling of cerebrospinal fluid in patients with aneurysmal subarachnoid hemorrhage

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OBJECTIVE MicroRNAs (miRNAs) regulate gene expression and therefore play important roles in many physiological and pathological processes. The aim of this pilot study was to determine the feasibility of extraction and subsequent profiling of miRNA from CSF samples in a pilot population of aneurysmal subarachnoid hemorrhage patients and establish if there is a distinct CSF miRNA signature between patients who develop cerebral vasospasm and those who do not.

METHODS CSF samples were taken at various time points during the clinical management of a subset of SAH patients (SAH patient samples without vasospasm, n = 10; SAH patient samples with vasospasm, n = 10). CSF obtained from 4 patients without SAH was also included in the analysis. The miRNA was subsequently isolated and purified and then analyzed on an nCounter instrument using the Human V2 and V3 miRNA assay kits. The data were imported into the nSolver software package for differential miRNA expression analysis.

RESULTS From a total of 800 miRNAs that could be detected with each version of the miRNA assay kit, a total of 691 miRNAs were communal to both kits. There were 36 individual miRNAs that were differentially expressed ($p < 0.01$) based on group analyses, with a number of miRNAs showing significant changes in more than one group analysis. The changes largely reflected differences between non-SAH and SAH groups. These included miR-204-5p, miR-223-3p, miR-337-5p, miR-451a, miR-489, miR-508-3p, miR-514-3p, miR-516-5p, miR-548 m, miR-599, miR-937, miR-1224-3p, and miR-1301. However, a number of miRNAs did exclusively differ between the vasospasm and nonvasospasm SAH groups including miR-27a-3p, miR-516a-5p, miR-566, and miR-1197.

CONCLUSIONS The findings indicate that temporal miRNA profiling can detect differences between CSF from aneurysmal SAH and non-SAH patients. Moreover, the miRNA profile of CSF samples from patients who develop cerebral vasospasm may be distinguishable from those who do not. These results provide a foundation for future research at identifying novel CSF biomarkers that might predispose to the development of cerebral vasospasm after SAH and therefore influence subsequent clinical management.

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KEY WORDS miRNA; subarachnoid hemorrhage; vasospasm; cerebrospinal fluid; vascular disorders

ANEURYSMAL subarachnoid hemorrhage (aSAH) involves extravasation of blood into the subarachnoid space secondary to aneurysm rupture. In Australia, the incidence of aSAH is estimated at 10.3 cases per 100,000 persons per year.²⁸ It has also been estimated that 450,000 to 1.4 million people may harbor unruptured aneurysms, with more than 2200 of these leading to an aneurysmal hemorrhage every year.¹⁰ The lowest incidence

reported around the world is 1.04 people per 100,000 population/year in the Middle East,²¹ and the highest incidence reported is 20 people per 100,000 population/year in Finland and Japan.^{20,25} The outcome for patients with aSAH is generally very poor as it is associated with a high mortality rate and an increased loss of productive years in patients younger than 65 years.⁵³

Cerebral vasospasm (CVS), characterized by a delayed

ABBREVIATIONS aSAH = aneurysmal SAH; CVP = central venous pressure; CVS = cerebral vasospasm; miRNA = microRNA; miRTarBase = microRNA-target interactions database; MTI = miRNA-target interaction; SAH = subarachnoid hemorrhage.

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ischemic neurological deficit and radiological evidence of cerebral vasoconstriction, is a common complication after aSAH. Of the 2200 Australian patients with an aSAH every year, approximately one-third of these people will be affected by symptomatic CVS, which can lead to the development of additional focal neurological deficits causing ischemic strokes and ultimately death. The precise mechanisms contributing to the development and progression of CVS remain unknown. Data generated from functional genomic studies are providing evidence that molecular regulatory processes contribute to the formation and rupture of aneurysms.^{33,35,44} However, there are no established circulating biomarkers that may be used to reliably predict the onset of CVS. MicroRNAs (miRNAs) are a class of evolutionary conserved, small, noncoding single-stranded RNA molecules of approximately 22 nucleotides in length, involved in the regulation of gene expression at a posttranscriptional level.¹¹ They modulate protein expression through base pairing with a complementary sequence in the 3'-untranslated region of messenger RNA (mRNA). The traditional outcome of miRNA is one of translational repression of gene expression; however, mRNA levels may also be reduced directly.³⁰ MicroRNAs have been shown to be involved in the regulation of multiple cellular processes including differentiation, proliferation, and apoptosis in both health and disease with bioinformatic studies proposing that miRNAs mediate the actions of up to one-third of all human genes.³¹ They have been reported to be stable⁸ and are expressed in abundance in the central nervous system, suggesting a substantial contribution to processes within the brain. Circulating miRNAs, packaged in microvesicles, have been detected in human serum and plasma as well as CSF³ and have been implicated in a range of central nervous system disorders including cancer,^{3,4} Alzheimer's disease,⁹ and multiple sclerosis.¹⁸ Expression patterns of miRNA have been shown to change over time in laboratory studies investigating ischemic stroke in rats.^{12,22} Also, a small study recently analyzed the cerebral microdialysate from 3 SAH patients and reported the presence of miRNA in brain interstitial fluid.²

The aim of this observational pilot study was 2-fold: 1) to determine the feasibility of extraction and subsequent profiling of miRNA from CSF samples in a population of aSAH patients, and 2) determine if there is a distinct CSF miRNA signature between patients who develop cerebral vasospasm and those who do not develop vasospasm following SAH.

Methods

Ethics Statement

This study was carried out with approval from The Royal Melbourne Hospital Ethics Committee in accordance with the NHMRC National Statement on Ethical Conduct in Human Research (2007). Informed consent was obtained for the collection of samples and subsequent analysis.

Patient Selection and Management

All patients were enrolled at The Royal Melbourne Hospital between February 2013 and November 2014. The

pilot study group comprised 23 patients (14 females and 9 males; mean age 52.7 years, median 54 years). Of the 23 patients, 9 patients had an aSAH with no vasospasm (4 females and 5 males; mean age 49.3 years, median 51 years), 10 patients with an aSAH and vasospasm (7 females and 3 males; mean age 54.2 years, median 56 years), and 4 control patients (3 females and 1 male; mean age 56.5 years, median 56 years) (Table 1).

The diagnosis of subarachnoid hemorrhage was confirmed using brain CT scanning or by the presence of blood/xanthochromia in the CSF collected via lumbar puncture. Symptomatic hydrocephalus was treated with insertion of a ventriculostomy catheter and drainage of CSF at the discretion of the treating neurosurgeon. All patients underwent cerebral digital subtraction angiography (DSA) for identification of aneurysm location and morphology. All aneurysms were secured either by endovascular coiling or microsurgical clipping within 24 hours of admission. Clinically significant intracerebral hematomas were evacuated. Postoperatively, patients were extubated and managed at a neurosurgical high dependency unit unless they required mechanical ventilation or inotrope support, in which case they were managed in the ICU.

All patients underwent insertion of a central venous catheter and were given supplementary fluids to maintain mild hypervolemia and a central venous pressure (CVP) target of greater than 8 cm H₂O. All patients were given prophylactic nimodipine (oral or intravenous) from admission and up to 21 days posthemorrhage. Hypertensive therapy was not initiated routinely but only after the diagnosis of cerebral vasospasm. Patients suspected of developing cerebral vasospasm who had a CVP lower than 8 cm H₂O were immediately given a fluid bolus (0.9% saline) to restore CVP to greater than 8 cm H₂O. If they remained symptomatic, hypertensive therapy was initiated using a noradrenaline infusion targeting a systolic blood pressure of up to 200 mm Hg or until neurological deficit reversal. Brain CT scanning was performed to exclude other causes of deterioration or an established infarct, and hypertensive therapy was continued in the ICU aiming to maintain the lowest possible systolic blood pressure at which the patient remained deficit free. Cerebral vasospasm was confirmed with cerebral angiography and in selected patients who remained symptomatic despite hypertensive therapy, balloon angioplasty or intraarterial nimodipine or verapamil were used at the discretion of the endovascular neuroradiologist and treating neurosurgeon.

Specimen Collection and Storage

For the length of time that the external ventricular drain remained patent and in situ, CSF was collected on a daily basis. For the participants included in this pilot study, the period ranged from 1 to 18 days (mean 6.9 days, median 6.1 days). The duration of drainage was determined entirely on clinical grounds, and the decision was made by the treating neurosurgeon. CSF was also collected from control patients via a lumbar puncture. These were patients who presented with sudden-onset headache and after exhibiting normal results on brain CT scanning had a lumbar puncture that excluded subarachnoid hemorrhage and no other cause for headache was identified. The samples

TABLE 1. Clinical profiles of patients included in the pilot study

Case No.	Sex	Age (yrs)	Admission CT Fisher SAH Grade	Admission WFNS Clinical Grade	Ruptured Aneurysm Location	Angiographic Spasm	Symptomatic Spasm	Symptomatic Spasm Day (post-SAH)	Sample Day	Hours Post-SAH
No SAH										
12	F	47	NA	NA	NA	NA	NA	NA	1	NA
18	M	71	NA	NA	NA	NA	NA	NA	1	NA
30	F	65	NA	NA	NA	NA	NA	NA	1	NA
34	F	43	NA	NA	NA	NA	NA	NA	1	NA
SAH/NV										
20	M	62	4	V	Basilar tip	No	No	NA	1	0.3
23	F	31	4	I	Lt pericallosal	No	No	NA	1	101.5
24	F	40	4	II	Rt A ₁	No	No	NA	1	12
25	M	54	4	III	ACoA	No	No	NA	7	173
38	F	40	4	V	Rt MCA	No	No	NA	1	42.5
38	F	40	4	V	Rt MCA	No	No	NA	8	181
39	M	54	4	III	ACoA	No	No	NA	7	164.5
40	M	69	4	II	Lt MCA	No	No	NA	2	53
41	F	43	4	II	Lt SCA	No	No	NA	1	21
42	M	51	4	I	Lt SCA	No	No	NA	2	98
SAH/CVS										
22	F	58	4	V	Lt Pericallosal	Mild	Yes	5	4	90
27	M	37	4	I	Rt MCA	Severe	Yes	3	1	215
32	F	76	4	I	Rt PCoA	Moderate/severe	Yes	7	1	102
33	F	36	4	II	ACoA	Severe	Yes	14	6	222
36	M	61	4	III	Lt SCA	Severe	Yes	14	1	385
44	F	54	4	I	Rt PCoA	Moderate/severe	Yes	10	1	264
45	F	51	4	II	ACoA	Moderate/severe	Yes	8	5	124
48	F	60	4	II	Basilar tip	Severe	Yes	11	6	147
52	F	50	4	IV	ACoA	Severe	Yes	11	1	433
53	M	59	4	IV	Lt vertebral	Mild	Yes	7	7	384

ACoA = anterior communicating artery; CVS = cerebral vasospasm; MCA = middle cerebral artery; NA = not applicable; NV = no vasospasm; PCoA = posterior communicating artery; SCA = superior cerebral artery; WFNS = World Federation of Neurosurgical Societies.

were collected and centrifuged at 2000g for 5 minutes at 4°C to remove any contaminating blood cells. Aliquots of the cell-free fractions were then stored in a -80°C freezer until further analysis.

miRNA Preparation

CSF samples were diluted 2:3 in RNase-free water and miRNA was purified using the miRcury RNA isolation kit—Biofluids (Exiqon) according to manufacturer's instructions. To enable recovery determination, samples were spiked with 2 µl of 0.1 µM ath-miR-159 and 0.01 µM osa-miR-414 after the lysis step. Samples were subsequently eluted in 100 µl RNase-free water. To remove contaminants, samples were diluted with 320 µl RNase-free water and concentrated to a volume of 30 µl using an ultra 0.5 centrifugal filter unit with an ultracel-3 membrane (Amicon).

Nanostring Expression and Statistical Analysis Selection of Candidate miRNAs

Three microliters of purified miRNAs was analyzed

using an nCounter instrument (Nanostring Technologies) using the Human V2 or V3 miRNA assay kit (Nanostring Technologies) according to the manufacturer's instructions. NanoString RCC (Reporter Code Counts) files were then imported into the nSolver Analysis software (V 2.6.43) for differential expression evaluation between comparison groups. The parameters for the statistical analysis of the groups in Table 2 were set as follows: a z-score transformation (z-score genes); Distance metric—Euclidean Distance; Linkage Method—Average; Sample/Gene Data—Clustering. The comparison groups that underwent Differential Expression Analysis with the nSolver Analysis software were as follows: Comparison 1, no SAH versus SAH/no vasospasm (combined); Comparison 2, no SAH versus SAH/no vasospasm (Sample Day 1); Comparison 3, no SAH versus SAH/vasospasm (combined); Comparison 4, no SAH versus SAH/vasospasm (Sample Day 1); Comparison 5, no SAH versus SAH/vasospasm (post Sample Day 1); Comparison 6, SAH/no vasospasm (combined) versus SAH/vasospasm (combined); Comparison 7, SAH/no vasospasm (Sample Day 1) versus SAH/vasospasm (Sample

TABLE 2. miRNA-predicted target genes (miRTarBase v6.0) analysis for miRNA with (p < 0.01) fold change as determined by nSolver analysis

miRNA	Comparisons Where Significant	Fold Change*	Strong Evidence Target Genes	Weak Evidence Target Genes
hsa-miR-26a-5p	1	-1.53	52	378
hsa-miR-27a-3p	7	1.34	39	358
hsa-miR-137	3	-1.75	28	96
hsa-miR-152	1	-1.79	18	151
hsa-miR-204-5p	1, 3	3.15, 2.79	49	337
hsa-miR-223-3p	1, 3	2.95, 5.55	41	46
hsa-miR-301a-3p	2	-1.72	7	359
hsa-miR-302a-3p	5	-1.61	15	417
hsa-miR-320e	3	3.11	—	47
hsa-miR-337-5p	3, 4, 7	-1.24, -1.21, -1.41	—	3
hsa-miR-345-5p	3,5	-1.88, -1.95	3	38
hsa-miR-346	3	-1.65	6	77
hsa-miR-376c	1	-1.47	5	106
hsa-miR-378d	2	-2.32	—	26
hsa-miR-421	1	-1.47	4	218
hsa-miR-451a	1, 3, 5	72.01, 59.5, 108.64	15	9
hsa-miR-489	3,5	-1.59, -1.93	1	61
hsa-miR-508-3p	3, 6	-1.85, -1.61	—	50
hsa-miR-514b-3p	3	-1.99	—	52
hsa-miR-516-5p	8	-1.69	—	—
hsa-miR-519b-3p	3, 4, 6	-1.69, -1.73, -1.53	2	317
hsa-miR-521	3	-1.74	1	21
hsa-miR-548m	1, 3, 4	-1.42, -1.64, -1.7	1	90
hsa-miR-566	6	-1.36	—	52
hsa-miR-599	1, 3	-1.53, -1.9	—	101
hsa-miR-606	1	-1.43	—	57
hsa-miR-624-3p	3	-1.49	1	75
hsa-miR-626	1	-1.88	—	32
hsa-miR-708-5p	3	-1.76	17	60
hsa-miR-937	3, 5	-1.81, -2.00	—	88
hsa-miR-1197	8	-1.48	—	48
hsa-miR-1224-3p	1, 3, 5	-2.08, -2.61	—	244
hsa-miR-1301	1, 3	-1.72	—	20
hsa-miR-2117	3	-1.3	—	49
hsa-miR-3180-5p	1	-1.52	—	124
hsa-miR-4521	3	-1.92	—	25

— = no current experimental evidence.

* Fold-change figures in order of group comparisons where a significant miRNA difference was detected.

Day 1); and Comparison 8, SAH/no vasospasm (post Sample Day 1) versus SAH/vasospasm (post Sample Day 1) (see Fig. 1 and Supplementary Table 1).

miRNA-Target Interactions

Target genes for each candidate miRNA were subsequently retrieved from miRTarBase (V6.0) (mirtarbase.mbc.nctu.edu.tw).¹⁹ The miRNA target genes included in this database are all experimentally validated in other studies and are published in the literature. In the 2015 mir-

TarBase update, 324,219 human miRNA-target interactions (MTIs) were collected between 2619 miRNAs and 12,738 target genes with experimental support from 4264 articles (including 3527 and 5081 interactions confirmed by Western blot and reporter assays, respectively). The miRTarBase database utilizes a text-mining system that surveys published literature which describes MTIs that have been verified by various experimental methods. These results are further scrutinized by the database developers to confirm the experimental validation of the target genes.

FIG. 1. Schematic flow diagram of pilot study protocol to identify putative target genes of the differentially expressed CSF miRNA.

Results

miRNA Microarray Analysis and Screening

The miRNA expression analysis was undertaken by analyzing CSF from the patients listed in Table 1. The miRNA expression profile of 800 miRNAs was determined using the nCounter Human V2 and V3 miRNA Expression Assay kits (nanoString Technologies). Sample preparation using the kits involves the multiplexed annealing of specific tags to the target miRNAs followed by a ligation reaction, enzymatic purification step and the inclusion of control RNA for monitoring ligation efficiency and specificity. Amplification steps are omitted from this protocol to prevent the introduction of bias into the results. A schematic flow diagram of the pilot study protocol for identifying putative target genes of the differentially expressed CSF miRNA is shown in Fig. 1.

In our pilot study, miRNAs were detected in the CSF samples from all patients. The patient comparison groups that were analyzed in this pilot study are identified in Fig. 1 and comprehensively listed in Supplementary Table 1. The miRNAs that were differentially expressed and determined to be statistically significant are listed in Supplementary Table 2. The conditions used in this approach with the nSolver analysis software were as follows: a z-score transformation (z-score genes); Distance metric—Euclidean Distance; Linkage Method—Average; Sample/Gene Data—Clustering. A total of 265 miRNAs were differentially expressed at a significance level of $p < 0.05$ (Supplementary Table 2). Of these, 36 miRNAs exhibited fold change with a significant ($p < 0.01$) difference (Supplementary Table 2) and we undertook further examination to determine their target genes.

The target genes for each candidate miRNA were identified from miRTarBase with reference to experimentally validated human miRNA target interactions (Supplementary Table 3). A summary list of validated target genes was recovered for each of the 36 differentially expressed miRNAs, which was divided into miRNA target gene interactions with strong and weak evidentiary data. Table 2 indicates the number of strong and weak evidence target genes for each of the miRNA. Of these, 19 miRNAs were observed to currently have strong experimental validation for their listed target genes in miRTarBase, including miR-26a-5p, miR-27a-3p, miR-137, miR-152, miR-204-5p, miR-223-3p, miR-301a-3p, miR-302a-3p, miR-345-5p, miR-346, miR-376c, miR-421, miR-451a, miR-489, miR-519b-3p, miR-521, miR-548 m, miR-624-3p, and miR-708-5p. These miRNA and their target genes with strong evidentiary experimental support are listed in Supplementary Table 4.

It is interesting to note that when assessing the 36 miRNAs ($p < 0.01$) that were found to be significantly differentially expressed, 18 were observed as a unique miRNA located within a single comparison group. These included miR-301a-3p, miR-378d (Comparison 2), miR-137, miR-320e, miR-346, miR-514-3pm, miR-521, miR-624-3p, miR-708-5p, miR-1244, miR-2117, miR-4521 (Comparison 3), miR-302a-3p, miR-548I (Comparison 5), miR-566 (Comparison 6), miR-27a-3p (Comparison 7), and miR-516a-5p, miR-1197 (Comparison 8). However, miR-451a showed significant increased fold changes in a number of group comparisons—72-fold (Comparison 1), 59-fold (Comparison 3), 39-fold (Comparison 4, at $p < 0.05$ only), and 108-fold (Comparison 5). All of these comparisons were

between non-SAH and SAH groups, indicating a potential major regulatory role of this miRNA in the pathophysiological processes involved in aSAH. Notably, Comparisons 3, 4, and 5 were all relative to the vasospasm patients. Specifically, the miRNA identified in Comparisons 6, 7, and 8, miR-566, miR-27a-3p, miR-516a-5p, and miR-119 indicated differences between the nonvasospastic and vasospastic patients within the aSAH group. Of these, only miR-27a-3p, which was differentially expressed on Day 1 post-aSAH, has genes with strong evidentiary experimental support.

The collected “strong evidence” MTIs in miRTarBase are validated experimentally by reporter assays, Western blot, and qPCR (quantitative polymerase chain reaction) and the weak evidence MTIs via the following methodology: microarray, NGS, and pSILAC.¹⁹ A search was then undertaken on the Web-based interface of GeneCards (www.genecards.org) to list known functions of the identified target genes from miRTarBase V6.0. Supplementary Table 5 identifies the function of strong evidentiary genes for 2 miRNAs, in particular, miR-27a-3p and miR-451a, which were differentially expressed in the specific comparisons. Thus, miR-451a may give insight into the processes underlying aSAH and the vasospastic state and miR-27a-3p might identify patients who could potentially develop vasospasm after aSAH.

Discussion

Cerebral vasospasm, characterized by a delayed ischemic neurological deficit and radiological evidence of cerebral vasoconstriction, is a common complication after aSAH, which contributes to significant added morbidity and mortality.^{24,32,34,49,56} The molecular basis and pathophysiology are still poorly understood, and a number of approaches have been used over many years to identify potential biomarkers that may be predictive factors in the clinical setting; however, further conclusive studies are still required.^{1,5,26,27,47} An excellent review by Jordan and Nyquist²³ outlines the approaches undertaken, based on the theoretical understanding of vasospastic mechanisms, to uncover potential biomarkers of clinical importance. While it is beyond the scope of this paper to discuss all of these areas, various indicators of vasospasm have been proposed. These include inflammatory molecules, endothelial activation, hemoglobin breakdown products, calcium metabolism in smooth muscle, nitric oxide donors, endothelin intervention and the coagulation cascade associated with SAH.²³ In addition, with advances in proteomic technologies, new protein candidates have also been suggested as potential biomarkers of vasospasm onset, including S100-B,⁴² s-GFAP,⁴¹ and NfH SMI35.⁴³

As the majority of the human genome consists of non-protein coding regions involving RNA genes, in particular miRNA, the potential role of miRNA in a number of human diseases has been explored over the last few years. Initially, miRNAs were implicated in the pathogenesis of cancer,^{29,51} but they have also been linked to neurodegenerative disease,^{15,50} cardiovascular disease,^{14,45} and stroke.^{46,57} In addition, the stability of miRNA detected in a number of different biological samples (serum, plasma, whole blood, and CSF) identifies them as possible biomarkers to be used in the early clinical intervention of patients, as

their role in the posttranscriptional regulation of gene expression makes them prospective therapeutic targets.²⁹ In this pilot study, we identified a number of differentially expressed miRNA in the CSF of patients with SAH. The utilization of the Human V2/V3 miRNA assay kits allows for the unbiased screening of a potential 800 miRNA in biological samples.

It is noteworthy that the expression levels of a number of miRNAs instead of a single miRNA, showed significant changes ($p < 0.01$) between various comparison groups in this study. The miRNAs regulated in common by the various conditions might provide insight into central mechanisms that precede cerebral vasospasm. We detected the differential expression of 9 miRNA (miR-26a-5p, miR-152, miR-269-5p, miR-376c, miR-421, miR-606, miR-626, miR-1301, and miR-3180-5p) in SAH patients who did not undergo cerebral vasospasm (Comparison 1), when matched to 16 miRNA (miR-137, miR-320e, miR-337-5p, miR-345-5p, miR-346, miR-489, miR-508-3p, miR-514b-3p, miR-519-3p, miR-521, miR-624-3p, miR-708-5p, miR-937, miR-1244, miR-2117, and miR-4521) in patients who underwent cerebral vasospasm after aSAH (Comparison 3). In addition, analysis of the first CSF samples (Sample Day 1) from aneurysmal patients experiencing vasospasm (Comparison 4) showed significant changes in expression levels of miR-337-5p, miR-519b-3p, and miR-548m. Finally, miR-516a-5p and miR-1197 were significantly reduced in CSF samples taken post-Day 1 for SAH patients who experienced cerebral vasospasm (Comparison 8).

Mature miRNA regulates protein transcription post-transcriptionally, predominantly by binding to complementary coding sequences on many different mRNAs, which can subsequently lead to translational repression¹⁶ or activation.⁵⁴ There are a number of databases available which use a number of prediction tools allowing for the identification of potential target genes. A study by Friedman et al.¹⁷ shows that of the 1000 currently known human miRNAs in their analysis, there is the potential for binding to hundreds of mRNA targets and subsequently affecting the expression of various genes. This is further supported in the summary of miRTarBase (Table 4), where 2619 human miRNAs have experimental evidence for 324,219 miRNA target interactions, which ultimately can influence the expression of 12,738 target genes. The miRNAs identified in our current pilot study also target a number of different genes as listed in Supplementary Table 5. However, we will only make reference to a select number of the target genes that have already been implicated in SAH.

As mentioned earlier, a number of mechanisms have been proposed as mediators of cerebral vasospasm post-SA.^{23,38,40,55} These include the upregulation of vascular inflammation, endothelial dysfunction and inhibition of the nitric oxide pathway in vascular smooth muscle, increased production of oxidative stress, and free radical and proinflammatory products. It has been previously shown that the expression of miRNAs is essential for the regulation of the vascular smooth muscle phenotype. Reduced levels of miR-451a in stretched versus nonstretched veins were linked to the phosphorylation of AMPK pathway components, suggesting a role in the promotion of vascular smooth muscle cell differentiation and contractile events.⁵²

In our study we observed lower CSF levels of miR-451a in patients who experienced SAH with vasospasm (Comparison 3, Day 1 samples) to patients who experienced SAH without vasospasm (Comparison 1). However, miR-451a levels were also observed to increase in CSF samples analyzed post-Day 1 (Days 4–7 post-SAH) from patients who experienced SAH with vasospasm (Comparison 5 vs Comparison 1), which may counteract the early onset of a vasospastic phenotype linked to the phosphorylation of AMPK pathway components.⁵² This further supports data from preclinical studies on the importance of miRNAs in regulating vascular smooth muscle cells in the vasospastic phenotype.³⁷

Additional experimentally validated targets of miR-451a (Supplementary Table 5) include the matrix metalloproteinases MMP-2 and MMP-9. MMP-2 and MMP-9 are gelatinases/collagenases that facilitate transport of immune cells to site of injury by degrading tight junctions and basal membrane proteins of the extracellular matrix. A study by McGirt et al.³⁶ analyzed the venous serum from patients admitted to hospital with SAH in an attempt to identify prognostic markers that could predict the occurrence of cerebral vasospasm. One of the molecules that were studied, MMP-9, was elevated in the serum prior to the development of cerebral vasospasm post-SAH. Along with vascular endothelial growth factor and von Willebrand factor, MMP-9 was not elevated in nonvasospastic SAH patients, indicating a potential role in cerebral vasospasm for these factors. As miR-451 has been shown to downregulate the expression of MMP-9 in lung cancer⁵⁸ and glioma cells,³⁹ the decrease in CSF miR-451a levels in patients who experienced SAH with vasospasm (Comparisons 3 and 4) to patients who experienced SAH without vasospasm (Comparison 1), may have also led to a decrease in MMP-9 levels prior to aneurysmal onset in our cohort. This is further supported by the observation that there is a reduction in CSF miR-451a expression in SAH patients experiencing vasospasm when comparing early samples (Sample Day 1) and later samples (post-Sample Day 1): Comparison 4 versus 5.

Another miRNA of particular interest was miR-27a-3p that was a unique miRNA identified in the early CSF samples of SAH patients who developed symptomatic vasospasm compared with the ones who did not (Comparison 7). miR-27a-3p is one of only 2 miRNAs differentially expressed between nonvasospastic and vasospastic SAH patients that have experimentally validated gene targets in miRTarBase. One of the validated gene targets of miR-27a-3p is p53. Endothelial cell apoptosis can occur in the vascular wall with cerebral vasospasm after SAH and p53 has been shown to be one of the central regulators.⁷ Also, the proliferation of smooth muscle cells is thought to contribute to the vasospastic phenotype during remodeling of the injured vascular wall post-SAH.⁵⁹ This is believed to be due to the intimal thickening and corresponding wall thickness that occurs during smooth muscle cell proliferation.⁶ It is known that p53 has multiple functions such as influencing cell cycle control and proliferation,¹³ including the induction of smooth muscle proliferation in restenotic human coronary arteries after percutaneous transluminal coronary angioplasty.⁴⁸

Although the main mechanism of miRNA action is known to be the downregulation of target genes, it has also been demonstrated that miRNAs may also contribute to the modulation of transcription or activation of translation, ultimately leading to the upregulation of target genes.^{12,54} Therefore, the observed increase in CSF levels of miR-27a-3p in our SAH patients with vasospasm may have led to a p53-mediated mechanistic induction of the vasospastic phenotype. A number of miRNA in the SAH patient Comparison groups 6, 7, and 8 were also observed to decrease in expression (miR-508-3p, miR-519b-3p, miR-566, miR-516-5p, and miR-1197), and it may be possible that one or more of these miRNA may also contribute to the vasospastic state (even though “strong experimental” evidence of target genes in miRTarBase was not available at this point of time).

In this study, we restricted our further analysis to miRNAs that were differentially expressed within the individual comparisons at $p < 0.01$. However, future studies could be extended into the miRNAs which were detected at $p < 0.05$. For example, miR-27a-3p ($p < 0.01$) is known to target p53; however, miRTarBase list the following miRNAs ($p < 0.05$) which can also target p53: miR-15a-5p (Comparisons 3 and 7), miR-10b-5p (Comparison 4), and miR-30a-5p (Comparison 8). This can be also be extended to miRNA for MMP-9: miR-491-5p (Comparison 2), miR-9-5p (Comparison 8), and miR-133b (Comparisons 3 and 5), indicating that a number of miRNAs with slightly less statistical significance in this study with similar target genes may be contributing to the onset of the vasospastic state.

Conclusions

As mature miRNAs regulate protein transcription by binding to complementary sequences on many different RNAs, it is known that a single miRNA will regulate several mRNAs and therefore possess a number of target genes. In our pilot study, we have detected significant changes in a number of miRNAs that might predispose the development of cerebral vasospasm after aSAH. Understanding the CSF miRNA profile, the functional effect of the target genes, and the corresponding regulatory network may assist in providing a screening tool for stratifying SAH patients for personalized treatment, prior to the onset and progression of vasospasm. However, this will require further research in larger patient cohorts to further validate our initial findings and determine predictive biomarker miRNA profiles for the clinical setting. Finally, the posttranscriptional regulation of gene expression carried out by miRNA also makes them attractive therapeutic targets. Laboratory-based research is currently being undertaken to develop miRNA antagonists or mimics that may selectively interfere with this miRNA–gene expression regulation. While the results presented in this pilot study are based on a limited sample size, it provides the foundation for this approach to be further explored.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Conception and design: Kaye, Stylli, Adamides, Ziogas. Acquisition of data: Kaye, Stylli, Adamides, Koldej, Ritchie, Ziogas. Analysis and interpretation of data: all authors. Drafting the article: all authors. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Kaye. Statistical analysis: Kaye, Stylli, Adamides, Luwor, Ziogas. Administrative/technical/material support: Stylli, Adamides, Koldej, Luwor, Ritchie, Ziogas. Study supervision: Kaye, Stylli, Adamides, Ziogas.

Supplemental Information

Online-Only Content

Supplemental material is available with the online version of the article.

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