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MicroRNA and gene expression changes in unruptured human cerebral aneurysms

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OBJECTIVE The molecular mechanisms behind cerebral aneurysm formation and rupture remain poorly understood. In the past decade, microRNAs (miRNAs) have been shown to be key regulators in a host of biological processes. They are noncoding RNA molecules, approximately 21 nucleotides long, that posttranscriptionally inhibit mRNAs by attenuating protein translation and promoting mRNA degradation. The miRNA and mRNA interactions and expression levels in cerebral aneurysm tissue from human subjects were profiled.

METHODS A prospective case-control study was performed on human subjects to characterize the differential expression of mRNA and miRNA in unruptured cerebral aneurysms in comparison with control tissue (healthy superficial temporal arteries [STA]). Ion Torrent was used for deep RNA sequencing. Affymetrix miRNA microarrays were used to analyze miRNA expression, whereas NanoString nCounter technology was used for validation of the identified targets.

RESULTS Overall, 7 unruptured cerebral aneurysm and 10 STA specimens were collected. Several differentially expressed genes were identified in aneurysm tissue, with *MMP-13* (fold change 7.21) and various collagen genes (*COL1A1*, *COL5A1*, *COL5A2*) being among the most upregulated. In addition, multiple miRNAs were significantly differentially expressed, with miR-21 (fold change 16.97) being the most upregulated, and miR-143–5p (fold change −11.14) being the most downregulated. From these, miR-21, miR-143, and miR-145 had several significantly anticorrelated target genes in the cohort that are associated with smooth muscle cell function, extracellular matrix remodeling, inflammation signaling, and lipid accumulation. All these processes are crucial to the pathophysiology of cerebral aneurysms.

CONCLUSIONS This analysis identified differentially expressed genes and miRNAs in unruptured human cerebral aneurysms, suggesting the possibility of a role for miRNAs in aneurysm formation. Further investigation for their importance as therapeutic targets is needed.

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KEY WORDS cerebral aneurysms; gene expression; microRNA expression; molecular mechanisms of disease; deep sequencing; vascular disorders

Cerebral aneurysm rupture is a devastating event with extremely high morbidity and mortality.[32] The molecular mechanisms behind cerebral aneurysm formation and rupture remain poorly understood. Some investigators have attempted to identify the genetic basis of their development. These studies have focused mainly on linkage analyses,[14,18] in cases of familial aneurysms, or association analyses[40] of single nucleotide polymorphisms. Most recently, limited gene expression studies[25,27,30,33–35] have identified genes (mainly related to inflammation and extracellular matrix remodeling) with differential expression in aneurysm tissue. These groups used microarray and polymerase chain reaction (PCR) techniques. Their results are not readily comparable due to the use of dif-

ABBREVIATIONS AAA = abdominal aortic aneurysm; ECM = extracellular matrix; FDR = false discovery rate; GO = Gene Ontology; miRNA = microRNA; PCR = polymerase chain reaction; P_{CT} = probability of conserved target; STA = superficial temporal artery; VSMC = vascular smooth muscle cell.

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ferent microarray platforms, gene nomenclatures, and control tissues.[34] While genome-wide analysis of gene expression using deep sequencing has not been performed yet, such data allow the quantification of the expression of both annotated and unannotated genes.

In the past decade, microRNAs (miRNAs) have been shown to be key regulators in a host of biological processes.[10] They are noncoding RNA molecules, approximately 21 nucleotides long, that posttranscriptionally inhibit mRNAs by attenuating protein translation and promoting mRNA degradation.[10] Experiments with vascular smooth muscle cell (VSMC)–specific Dicer depletion emphasized the importance of miRNAs for VSMC homeostasis, and it is therefore likely that miRNAs also play a prominent role in aneurysm formation.[2] It has been shown[2,12,17,26,28,29,31] that miR-29, miR-21, miR-26, miR-143, and miR-145 play pivotal roles in the formation of abdominal aortic aneurysms (AAA). These miRNAs posttranscriptionally regulate the expression of multiple targets with a function in extracellular matrix, such as collagen and fibrillin, and thus affect vascular wall stability and aneurysm formation.[2,12,17,26,28,29,31] The identification of the specific miRNAs implicated in cerebral aneurysms, and their association with gene expression, is particularly appealing because these molecules can be silenced, providing potential therapeutic targets. A recent study[20] investigating the miRNA profile of ruptured cerebral aneurysms lacked controls for multiple comparisons and a separate analysis of corresponding gene expression.

To address these shortcomings, we performed a prospective case-control study in human subjects to measure the gene and miRNA expression in unruptured cerebral aneurysm tissue. Gene expression was investigated with the use of whole-genome deep sequencing. In addition, we attempted to identify statistically significant miRNA-mRNA pairs with correlated expression changes that were consistent with miRNA regulation.

Methods

Study Design

The current study was approved by the Dartmouth Committee for the Protection of Human Subjects, and every participant signed an informed consent. We followed a prospective case-control design to investigate the expression profiles of miRNA and mRNA, as well as their correlations in unruptured human cerebral aneurysm tissue. Cerebral aneurysm samples were obtained from patients undergoing craniotomy for unruptured cerebral aneurysm clipping at our institution between 2012 and 2014.

Control tissue was collected from the superficial temporal artery (STA) of patients undergoing pterional craniotomies (for aneurysm clipping or other treatments) or patients undergoing temporal artery biopsies to rule out temporal arteritis (used only after the regular pathological analysis was negative for temporal arteritis). STA has been used in prior comparative studies of cerebral aneurysm tissue as a control.[34] Clinical histories were obtained from all patients preoperatively on the day of the surgery. No postoperative follow-up data were collected for the purpose of this study.

Specimen Collection

After successful clipping of the aneurysm, the dome was resected with the use of microscissors and bayonetted forceps (not used before during the case) and was placed immediately into a vial containing RNAlater solution (Ambion) to protect RNA from degradation. The vials were immediately transported to the Genomics and Molecular Biology Shared Resource in the Geisel School of Medicine at Dartmouth, and frozen to –80°C. The same process was followed for the control tissue.

RNA Isolation and Library Preparation

RNA was harvested from the patient samples using the mirVana miRNA isolation kit (Ambion), which allowed total RNA that contained the miRNA population to be isolated. RNA quality and quantity were assessed using the Fragment Analyzer (Advanced Analytical) and Qubit (Invitrogen), respectively. The mRNA-sequencing libraries were prepared from 100 ng of total RNA using the Ion Total RNA-seq kit v2 (Life Technologies).

RNA was fragmented prior to adaptor ligation, reverse transcription was performed, and the resulting cDNA was purified and amplified. This protocol allows for strand information to be preserved so that all mapped reads are aligned in the direction of transcription relative to the chromosomal strand. Each biological sample was generated with a unique INDEX sequence to allow multiple samples to be pooled together (the Ion Xpress RNA barcodes were provided by Life Technologies). Library quality was assessed using the Fragment Analyzer (Advanced Analytical) and Qubit (Invitrogen) prior to sequencing.

Ion Torrent Sequencing

RNA sequencing was performed on the Ion Proton sequencer (Life Technologies), using the PI chip, according to the manufacturer's protocol. The sequencing template was prepared from 6 pM of the pooled libraries (n = 3) by emulsion PCR using the One Touch 2 Instrument (Life Technologies) and the Ion PI Template OT2 200 kit v2 chemistry (Life Technologies). Following enrichment on the One Touch ES Instrument, the sample was loaded onto the PI chip and sequenced using Ion PI Sequencing v2 chemistry with the preexisting Ion RNA-whole transcriptome run plan utilizing 500 flows. The Proton server is preloaded with base-calling and adapter-trimming software. FASTQ files were generated and used for data analysis. On average (across the 17 samples), 24 million reads were generated for each barcoded biological sample, with a median read length of 90 base pairs.

The reads were aligned using TopHat (version 2.0.10), disallowing novel splice junctions, and statistically significant transcripts were identified by comparing gene expression in cerebral aneurysm and STA tissues with Cuffdiff (version 2.2.1) using each sample as a replicate and a false discovery rate (FDR) of 0.05. Gene Ontology (GO) terms that were statistically enriched in up- and downregulated genes at this level of significance were identified with GOEAST using a hypergeometric test and a Yekutieli FDR of 0.01.

miRNA Expression

Affymetrix miRNA 4.0 microarrays (Affymetrix) were used to analyze the miRNA expression levels from the same RNA isolates. Following the manufacturer's protocol, 130 ng of total RNA was labeled using the FlashTag Biotin HSR RNA labeling kit (Affymetrix). To confirm efficient labeling, the ELOSA QC assay (Affymetrix) was performed, and samples were hybridized on the Affymetrix 4.0 microarrays overnight. Washing, staining, and scanning of the arrays was performed using the Command Console, and the spike-in analysis of the control oligonucleotides was evaluated using the Expression Console. Data were analyzed using the recommended Transcriptome Analysis Console software from Affymetrix.

miRNA and mRNA expression were quantified and normalized for each patient so that they could be compared across patients. We quantified the differences between cerebral aneurysms and control tissues by computing the ANOVA p value for each miRNA across different individuals and using the Benjamini–Hochberg multiple test correction with an FDR of 0.15 to identify statistically significant changes in expression. An FDR of 0.15 was used to account for the inherent variability expected in clinical samples.

NanoString Validation

To validate the miRNA array expression data, the expression of approximately 800 miRNAs was determined using the NanoString nCounter Human v2 miRNA Expression Assay (NanoString Technologies) and 100 ng of total RNA as the input. The protocol was carried out as described in the nCounter miRNA Expression Assay manual and sample preparation and hybridization protocols (November 15, 2012 version). Incubations were performed in a Veriti 96-well Thermocycler (Applied Biosystems). The duration of the overnight hybridization step was 13 hours. Samples were processed and applied to the NanoString sample cartridge using the nCounter Prep Station, and data were collected using the nCounter Digital Analyzer.

The data generated in this study were submitted to the Gene Expression Omnibus under accession number GSE66240.

Results

Patient Characteristics

During the study period, a total of 7 samples of unruptured cerebral aneurysm tissue (mean age 47 years; range 17–68 years; 2 male subjects) and 10 samples of control tissue (mean age 63.4 years; range 50–77 years; 1 male subject) were collected. The collected aneurysms were from the following locations: 3 in the middle cerebral artery, 2 in the posterior communicating artery, 1 in the internal carotid artery terminus, and 1 in the pericallosal artery. The mean aneurysm size was 11 mm (range 4–25 mm).

Three of the control specimens were harvested from aneurysm patients, whose aneurysms were also collected, whereas 2 specimens belonged to aneurysm patients whose aneurysm domes were intraoperatively judged to be too small (mean size 4 mm) to be safely resected. Four of the control specimens were harvested during STA biopsies, which eventually were deemed nonpathologic on histology. The remaining control specimen was collected during a craniotomy for tumor resection. The relative vascular comorbidities of our 2 cohorts are detailed in Supplemental Table I.

Gene Expression Profile

We identified 1028 genes (Fig. 1) with differential expression between aneurysm and control tissue at an FDR of 0.05. From these, 623 were upregulated and 405 were downregulated. The top 20 upregulated and top 20 downregulated genes with the largest fold changes are listed in Tables 1 and 2. The protein-coding gene with the largest fold change in expression was a member of the matrix metalloproteinase family (*MMP-13*) (fold change 7.21), which codes for a metalloproteinase involved in extracellular matrix (ECM) remodeling and collagenase activity. We also identified a number of host genes for miRNAs, suggesting that some of the differential expression observed in the mature miRNAs could occur before processing by Dicer and Drosha. In particular, *MIR143HG*, the host gene for both miR-143 and miR-145, is downregulated in cerebral aneurysms with a highly significant 5-fold reduction, likely explaining their decreased expression. Among the most statistically enriched GO terms (Supplemental Table II) found in this data set were "extracellular matrix," "collagen metabolic process," and "response to stress."

miRNA Expression Profile

We identified 1338 miRNAs (Fig. 2) with differential expression between aneurysm and control tissue at an FDR of 0.15. Among these, 19 had a log fold change of 2 or greater, and 5 had a log fold change of less than –2. The 15 most upregulated and the 15 most downregulated miRNAs are presented in Table 3. The mature miRNAs with the largest absolute fold change were miR-21 (fold change 16.97) and miR-143–5p (fold change –11.14). The 3-prime product of miR-143, which has conserved target sites, was also significantly downregulated (fold change –5.27). Both miR-21 and miR-143 have been implicated in the pathogenesis of AAA. These results were confirmed with the use of NanoString technology, with results that were correlated with the original microarray results (Supplemental Figure I).

miRNA Regulatory Effects

We investigated our cohorts for pairs of miRNAs and their target genes that showed changes in expression that are consistent with miRNA regulation. We analyzed Targetscan Human Release 6.2, which consists of 72,770 miRNA family-gene pairs with annotated target sites in the 3' untranslated region (UTR) of at least 1 isoform. Since each miRNA family has multiple mature miRNA members, we had 165,560 distinct miRNA-gene pairs with measured expression data to investigate. Within this data set, there were 495 miRNA-gene pairs with predicted conserved target sites that satisfied both of the above-defined FDR thresholds. Furthermore, we required that the sites have a probability of conserved target (P_{CT}) of 0.25 or greater, which resulted in 370 miR-gene pairs: 68.7%

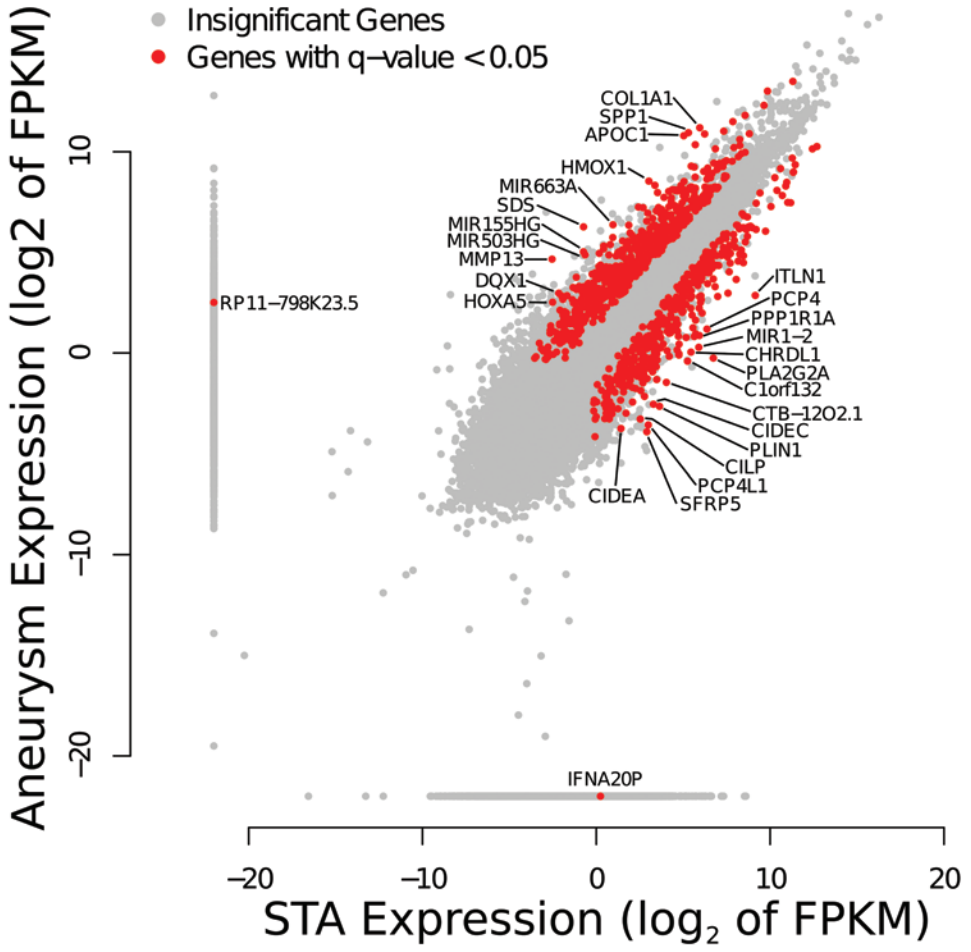

FIG. 1. Differential expression by RNA sequencing of the genes in unruptured cerebral aneurysms in comparison with STA tissues using Cuffdiff. Insignificant changes in gene expression are shown as *gray dots*; statistically significant changes in expression at an FDR of 0.05 are shown in *red*. Genes with a $\log_2$-fold change of 4 or greater are labeled. FPKM = fragments per kilobase of transcript per million fragments mapped. Figure is available in color online only.

of which showed opposite changes in expression that were consistent with microRNA regulation.[19]

We then examined the effect of highly differentially expressed miRNA with a fold change of 2 or greater in this data set. Only 5 microRNAs were differentially expressed at this level, targeting 17 distinct genes (Fig. 3). Most of these miRNAs have significantly differentially expressed target genes with opposite changes in expression in comparison with miRNA. The exceptions to this pattern are miR-10b and miR-181a. Although other regulatory effects could explain these differences, it is notable that both of these miRNAs have been shown to exhibit expression that is significantly correlated with their targets.[16] Graphs demonstrating the correlation of these miRNAs with the expression of their targets are presented in Supplemental Figures II, III, IV, V, and VI.

Specifically, miR-21 was upregulated with significant downregulation of its target gene *PAIP2B*, a translational inhibitor. miR-143 was downregulated with the significant upregulation of several of its targets, including multiple genes involved in the metabolism of collagen (*COL1A1*, *COL5A1*, and *COL5A2*), *MARCKS* (which encodes an actin filament cross-linking protein), and *TANC2*. miR-145 was also downregulated with the corresponding upregulation of *ABCA1*, *ADAMTS2*, and *BCAT1*. The significant differential expression of miR-21, miR-143, and miR-145 was corroborated by NanoString (Supplemental Figures VII and VIII).

We additionally examined the role of the strong conservation of miRNA target sites by requiring a P_{CT} of 0.75 or greater for the miRNA-genes pairs that matched our FDR significance thresholds. At this conservation level, 76.1% of all miRNA-gene pairs show opposite changes in expression. This list consisted of 83 target sites for 33 miRNA within 78 genes. The list of genes with 10 or more highly conserved target sites included only 5 genes, including *BCAT1*, *TANC2*, *ABCA1*, *SIK1*, and *FOXF2*. We found that all of the miRNAs targeting these genes showed opposite changes in expression, suggesting that the regulation of these genes could be the aggregate effect of a number of differentially expressed miRNAs. These results are summarized in Supplemental Table III.

Discussion

In this prospective study, we describe the gene and

TABLE 1. Differentially expressed genes: Significantly upregulated genes with the highest fold change

Gene Name	STA FPKM	Cerebral Aneurysm FPKM	Fold Change (CA/STA)	p Value	Q Value	Description
RP11-798K23.5	0	5.69579	Inf	5.00E-05	0.00257671	Processed pseudogene
MMP13	0.171024	25.309	147.9850781	0.0007	0.0196391	Matrix metallopeptidase-13 (collagenase-3)
SDS	0.600028	77.034	128.3840087	5.00E-05	0.00257671	Serine dehydratase
MIR155HG	0.599021	32.6115	54.4413301	5.00E-05	0.00257671	MIR155 host gene (nonprotein coding)
APOC1	32.6523	1776.04	54.39249302	5.00E-05	0.00257671	Apolipoprotein C-I
SPP1	39.359	1972.19	50.10772631	5.00E-05	0.00257671	Secreted phosphoprotein 1
MIR503HG	0.638055	29.3629	46.01938704	0.0026	0.049529	MIR503 host gene (nonprotein coding)
HMOX1	8.13042	371.704	45.7176874	5.00E-05	0.00257671	Heme oxygenase (decycling)-1
MIR663A	1.94222	82.861	42.66303508	5.00E-05	0.00257671	miRNA-663a
COL1A1	61.7918	2337.76	37.83285161	5.00E-05	0.00257671	Collagen, type I, alpha 1
DQX1	0.22374	7.84051	35.04295164	0.0007	0.0196391	DEAQ box RNA-dependent ATPase 1
HOXA5	0.174839	5.74981	32.88631255	0.00185	0.0391237	Homeobox A5
RGS1	10.3331	322.062	31.16799412	5.00E-05	0.00257671	Regulator of G-protein signaling-1
SLC16A10	0.450357	13.573	30.13831249	0.00015	0.00623662	Solute carrier family-16 (aromatic amino acid transporter), member-10
KIAA1199	1.34223	39.8339	29.67740253	5.00E-05	0.00257671	KIAA1199
GREM1	0.249676	7.14698	28.62501802	5.00E-05	0.00257671	Gremlin-1, DAN family BMP antagonist
ACP5	5.36724	152.303	28.37640948	5.00E-05	0.00257671	Acid phosphatase-5, tartrate-resistant
APOC2	1.92664	53.409	27.72131794	5.00E-05	0.00257671	Apolipoprotein C-II
PLA2G7	1.12637	30.951	27.47853725	5.00E-05	0.00257671	Phospholipase-A2, group VII (platelet-activating factor acetylhydrolase, plasma)

CA = cerebral aneurysm; Inf = infinity; FPKM = fragments per kilobase of transcript per million fragments mapped.

TABLE 2. Differentially expressed genes: Significantly downregulated genes with the highest absolute fold change

Gene Name	STA FPKM	Cerebral Aneurysm FPKM	Log Fold	Q Value	Description
IFNA20P	1.18	0.00	–inf	2.26E-02	Interferon, alpha-20, pseudogene
PLA2G2A	106.23	0.84	–6.98	2.58E-03	Phospholipase-A2, group IIA (platelets, synovial fluid)
SFRP5	7.50	0.07	–6.82	2.95E-02	Secreted frizzled-related protein-5
PCP4L1	7.94	0.08	–6.56	2.87E-02	Purkinje cell protein 4-like-1
PLIN1	12.42	0.16	–6.28	7.63E-03	Perilipin 1
ITLN1	565.18	7.26	–6.28	2.58E-03	Intelectin-1 (galactofuranose binding)
CIDEC	9.74	0.17	–5.83	2.58E-03	Cell death-inducing DFFA-like effector c
CILP	5.76	0.10	–5.81	2.58E-03	Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase
C1orf132	37.97	0.76	–5.65	2.58E-03	Chromosome 1 open reading frame-132
MIR1-2	59.43	1.22	–5.61	2.58E-03	miRNA 1–2
CTB-12O2.1	16.33	0.36	–5.49	2.58E-03	Uncharacterized noncoding RNA
CHRDL1	43.49	1.03	–5.40	2.58E-03	Chordin-like-1
PCP4	82.39	2.28	–5.18	2.58E-03	Purkinje cell protein-4
CIDEA	2.67	0.07	–5.17	1.75E-02	Cell death-inducing DFFA-like effector a
PPP1R1A	61.02	1.83	–5.06	2.58E-03	Protein phosphatase-1, regulatory (inhibitor) subunit 1A
FAM180B	6.82	0.22	–4.93	2.58E-03	Family with sequence similarity-180, member B
CCL21	26.95	0.93	–4.86	2.58E-03	Chemokine (C-C motif) ligand-21
SBSPON	49.80	1.76	–4.82	4.50E-03	Somatomedin B and thrombospondin, type 1 domain containing
MIR4453	27.00	0.97	–4.80	2.58E-03	miRNA-4453
AZGP1	11.11	0.40	–4.79	2.58E-03	Alpha-2-glycoprotein-1, zinc-binding

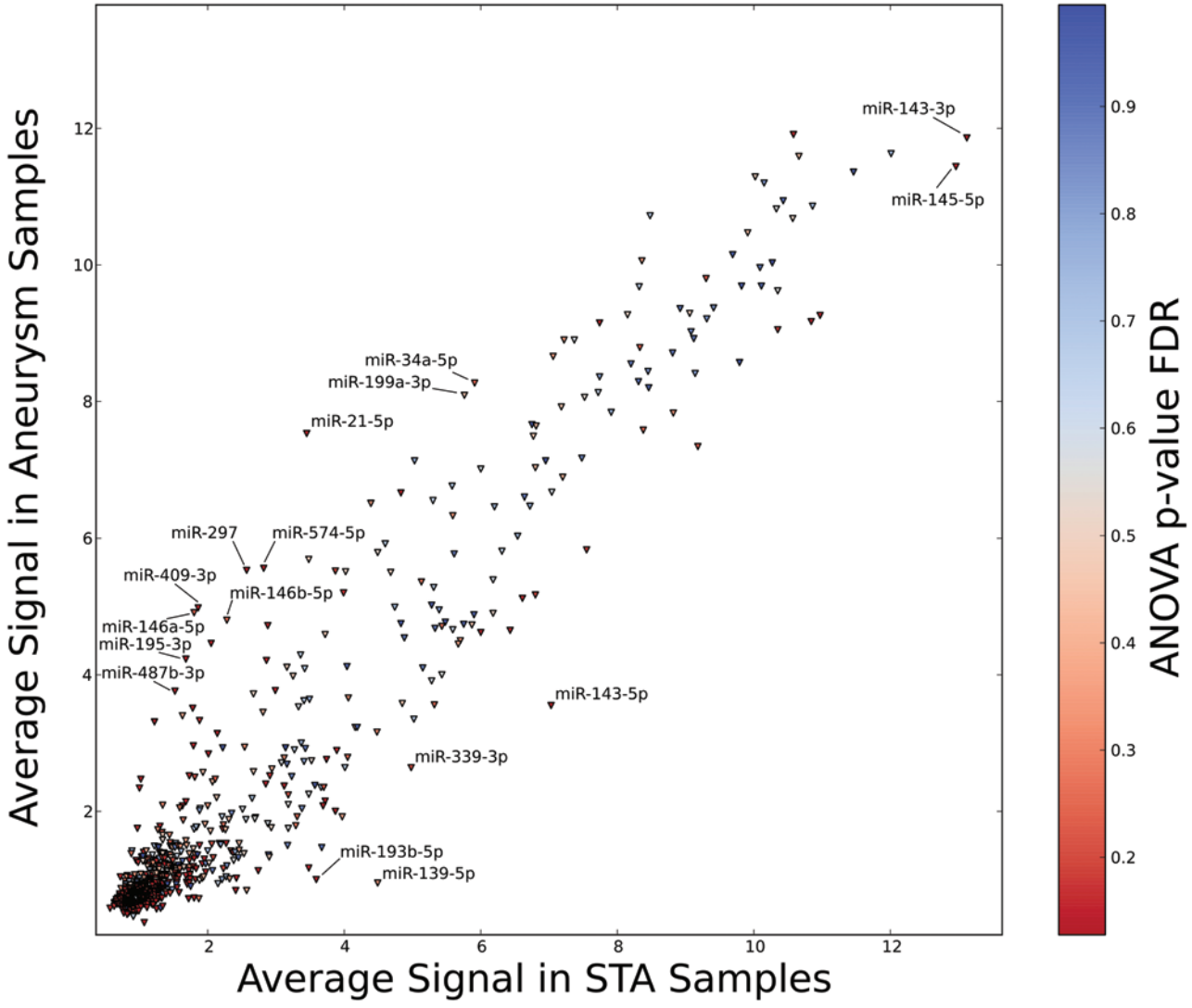

FIG. 2. Differential expression of miRNAs with annotated targets by microarray. Each *triangle* corresponds to a miRNA and is shaded by a color corresponding to the FDR at which it is significant. Highly significant miRNAs are labeled. Figure is available in color online only.

miRNA expression in unruptured cerebral aneurysm tissue in human subjects who underwent surgical clipping. Additionally, we detected gene-miRNA pairs with anticorrelated expression changes that were consistent with miRNA regulation. Our analysis addresses the methodological drawbacks of prior studies and utilizes deep sequencing to investigate the gene profile of unruptured human cerebral aneurysm tissue for the first time. Although changes in aneurysmal gene expression have been identified in microarray studies, the role of miRNAs in these changes is unknown. These molecules posttranscriptionally regulate mRNAs with complementary seed matches and hence could play a significant role in gene regulation.[10] In addition, miRNAs are highly conserved among eukaryotic organisms, making them ideal for reverse translational research in animal models of disease.

We identified a large number of differentially expressed genes in aneurysmal tissue. The most upregulated protein-coding gene was *MMP-13*, and several members of this family have been implicated[6,7,21,22,36,37] in the ECM remodeling of intracranial blood vessels. This results in the loss of structural support and a higher propensity for cerebral aneurysm formation. Additionally, this process can be mediated by most other highly upregulated genes in our cohort, which were involved in inflammatory regulation, ECM remodeling (*SPP1*), lipid metabolism (*APOC1*), and collagen formation (*COL1A1*, *COL5A1*, *COL5A2*, and others).[3–5,8,9,24] Several members of the collagen family were overexpressed, some of which have been identified in prior microarray studies.[25,27,30,35] This is likely a response to the degradation of ECM and thinning of the arterial wall, resulting in fibrosis. The downregulated genes in our cohort had a complementary effect on the overexpressed ones, with potential roles in defective ECM (*CILP*), inadequate lipid metabolism and storage (*ITLN1*, *PLIN1*, and its target *CIDEC*), as well as defective apoptosis (*CIDEC*, *SFRP5*), and intracellular signaling (*PLA2G2A*).

The miRNA expression profiles of the cerebral an-

TABLE 3. Significantly differentially expressed miRNAs with the highest fold changes

Mature miRNA Name	Aneurysm Signal	STA Signal	Fold Change	ANOVA p Value	ANOVA FDR
Upregulated					
miR-21-5p	7.53	3.45	16.97	0.075185	0.139337
hsa-miR-1246	5.86	2.36	11.26	0.005104	0.127714
hsa-miR-6875-3p	5.87	2.43	10.81	0.001077	0.127714
hsa-miR-6753-3p	5.59	2.37	9.28	0.00051	0.127714
hsa-miR-4685-3p	5.32	2.17	8.88	0.000825	0.127714
hsa-miR-409-3p	4.98	1.86	8.68	0.004163	0.127714
hsa-miR-297	5.53	2.57	7.75	0.000597	0.127714
hsa-miR-4484	8.84	5.98	7.24	0.000192	0.127714
hsa-miR-1972	4.8	2.03	6.85	0.000263	0.127714
hsa-miR-574-5p	5.56	2.82	6.69	0.003852	0.127714
hsa-miR-6877-3p	6.47	3.92	5.86	0.007492	0.127714
hsa-miR-487b-3p	4.23	1.68	5.85	0.022793	0.127714
hsa-miR-619-5p	8.87	6.35	5.72	0.00016	0.127714
hsa-miR-195-3p	4.46	2.05	5.33	0.000684	0.127714
hsa-miR-4793-3p	4.61	2.24	5.16	0.000186	0.127714
hsa-miR-4440	5.37	3.07	4.93	0.000441	0.127714
hsa-miR-382-5p	3.76	1.52	4.72	0.009787	0.127714
hsa-miR-432-5p	3.31	1.22	4.25	0.010858	0.127714
hsa-miR-3197	4.65	2.64	4.04	0.013788	0.127714
hsa-miR-127-3p	6.66	4.83	3.55	0.014006	0.127714
Downregulated					
hsa-miR-143-5p	3.55	7.03	11.14	0.006126	0.127714
hsa-miR-3195	2.09	5.08	–7.93	0.007712	0.127714
hsa-miR-6068	3.5	6.18	–6.39	0.00964	0.127714
hsa-miR-193b-5p	1	3.59	–6.01	0.016744	0.127714
hsa-miR-6848-5p	2.34	4.61	–4.81	0.058811	0.130779
hsa-miR-4655-5p	1.95	3.83	–3.67	0.072579	0.137742
hsa-miR-606	2	3.87	–3.64	0.007971	0.127714
hsa-miR-3613-3p	10.58	12.39	–3.51	0.014591	0.127714
hsa-miR-4445-3p	2.47	4.23	–3.39	0.02922	0.127714
hsa-miR-320a	9.26	10.97	–3.26	0.069366	0.135963
hsa-miR-5010-5p	0.95	2.64	–3.21	0.037616	0.127714
hsa-miR-320b	9.17	10.84	–3.19	0.088324	0.148826
hsa-miR-139-3p	1.13	2.74	–3.06	0.001682	0.127714
hsa-miR-933	2.08	3.69	–3.06	0.014096	0.127714
hsa-miR-940	2.15	3.72	–2.97	0.028514	0.127714
hsa-miR-452-5p	0.84	2.41	–2.96	0.02465	0.127714
hsa-miR-4743-5p	1.48	3.02	–2.9	0.072175	0.137684
hsa-miR-1468-3p	1.73	3.25	–2.86	0.003147	0.127714
hsa-miR-145-5p	11.44	12.96	–2.86	0.040347	0.127714
hsa-miR-6889-5p	1.39	2.91	–2.85	0.059015	0.130779

eurysms were also investigated. To limit our analysis to functionally significant miRNA, which results in post-transcriptional mRNA degradation, we investigated our sample for anticorrelated miRNA-mRNA pairs. miR-21 demonstrated the highest expression in our cohort, and was associated with multiple downregulated target genes, with *PAIP2B* (a translation repressor) being the most significant. Overexpression of miR-21 was identified before in human subjects and animal models of AAA, but not in human cerebral aneurysms.[1,11,23,28,38] There is controversy in regard to its role in AAA pathogenesis. Some animal AAA models[28] have demonstrated that upregulation of miR-21 results in protective cellular proliferation in an attempt to repair the defective vascular wall. Others[1,11,23,38] have supported that miR-21 contributes to AAA formation through the downregulation of ECM-remodeling genes.

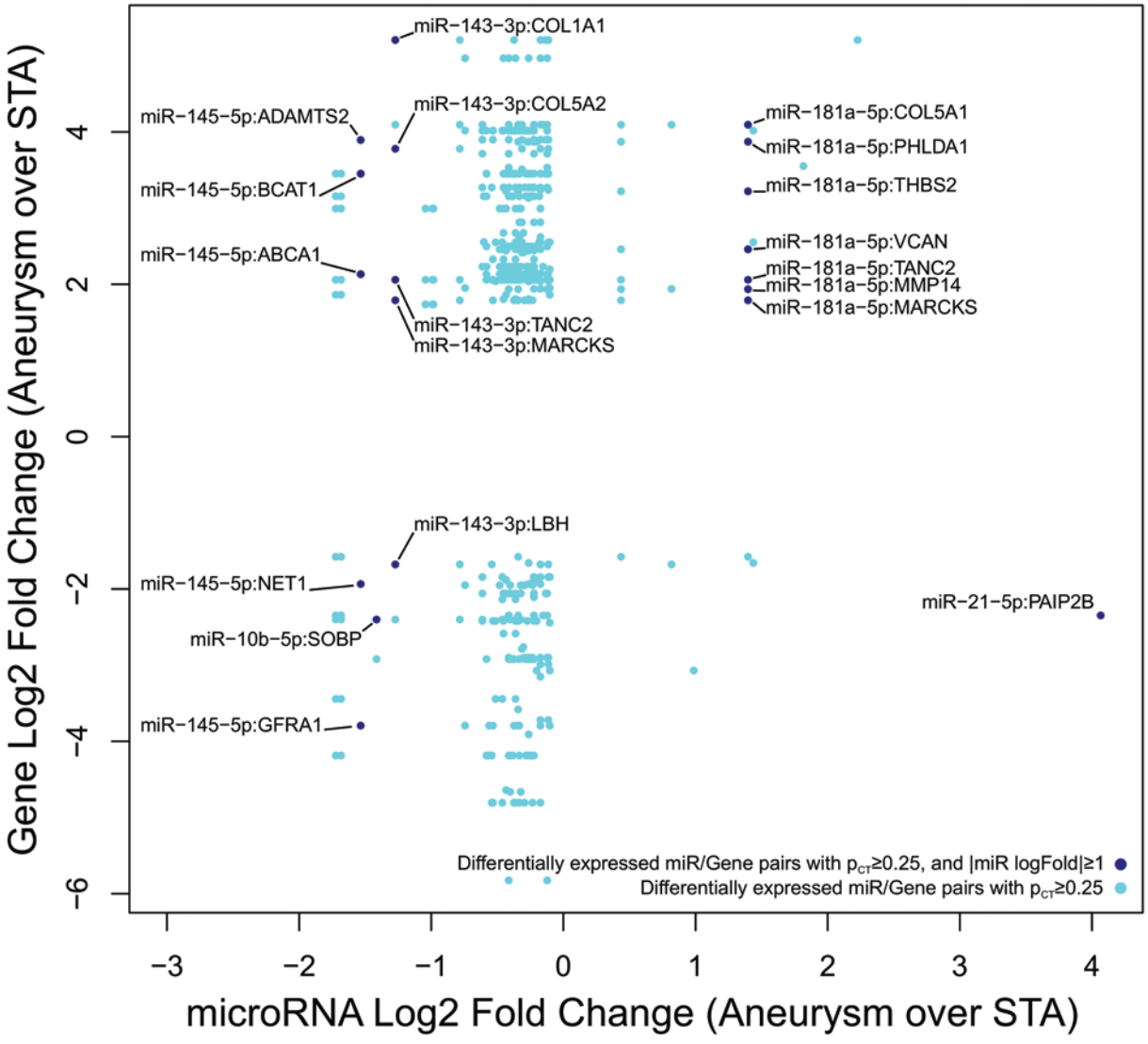

FIG. 3. The most significant miRNA-gene pairs. Each *dot* corresponds to a miRNA-gene pair, where *dark blue* corresponds to pairs with a miRNA fold change of 2 or greater, and *cyan* indicates all other pairs. Each column of dots with the same x-value corresponds to a single miRNA. At this level of significance, the majority of targeted genes have opposite changes in expression in comparison with the targeting miRNA. Figure is available in color online only.

Given the differences in pathophysiology between AAA and cerebral aneurysms, further research to unravel the role of miR-21 in the latter is needed.

miR-143 is the most significantly downregulated miRNA in our patients and has 3 upregulated target collagen genes, along with *MARCKS* and *TANC2*. Additionally, miR-143 downregulation has been identified as a central step that contributes to VSMC phenotypic modulation, which is crucial to cerebral aneurysm formation.[11,38] During this process, the VSMC loses its contractile apparatus and acquires a synthetic phenotype. As a result, they dissociate from each other and migrate into the intima, producing myointimal hyperplasia.[11,38] Several authors have demonstrated that this process results in the loss of structural integrity of the media, which is the layer that provides support to the vessel wall.[11,38] The downregulation of miR-145 plays a complementary role in VSMC homeostasis[13,15,17,39] and has been implicated in the pathophysiology of AAA in prior studies.[11,38] In addition, the downregulation of miR-145 has 3 significant upregulated targets with potential involvement in the pathophysiology of cerebral aneurysms. ADAMTS2 is a metalloproteinase involved in collagen synthesis and ECM remodeling. BCAT1 contributes to cellular proliferation, and ABCA1 is a cholesterol efflux regulatory protein. ABCA1 and BCAT1 also had highly conserved target sites for more than 10 distinct miRNAs, which are all downregulated in expression, suggesting that other miRNAs could also play a role in their upregulation.

A prior investigation of the miRNA expression patterns by Jiang et al.[20] focused on ruptured cerebral aneurysms, contrary to our study that investigated only unruptured aneurysms. The generalized inflammation associated with aneurysm rupture and subarachnoid hemorrhage might mask the tissue miRNA expression patterns and make it difficult to recognize which changes contribute to inflammation and which are part of the aneurysm formation

cascade. To address this, we elected to focus on unruptured aneurysms only. miR143 and miR145 were found to be downregulated by Jiang et al.,[20] in agreement with our study. However, they did not identify any upregulated miRNAs, contrary to our investigation. Jiang et al.[20] did not demonstrate anticorrelated pairs of miRNA and mRNA in order to identify functionally significant miRNAs. This limitation was addressed in our study.

The current study has several limitations. First, the selection of STA as the control tissue is not ideal. Although both the STA and the intracranial vessels arise from the same parent artery (common carotid artery), extracranial vessels can potentially have different baseline genetic profiles (given the presence of adventitia) in comparison with intracranial vessels. However, collecting control tissue from normal intracranial vessels is not possible, and STA is a well-established control in the prior literature.[34,35] In an attempt to further refine this control tissue, we used specimens not only from patients with aneurysms, but also from patients with unrelated intracranial pathology or no pathology at all.

Second, the present observational study design cannot establish if the identified profiles resulted in aneurysm formation or were an epiphenomenon, secondary to the presence of the aneurysm. In addition, we cannot identify the crucial molecular pathways the inhibition of which could halt or reverse aneurysm progression. However, the current data create a framework for further investigation in an animal model. The first step in this process would involve the creation of genetically modified endothelial cell lines, where the identified miRNA targets would be up- or downregulated in an effort to identify the downstream effects of these interventions and determine if the latter agree with our study. Subsequently, we would use rodent cerebral aneurysm models, where we would silence upregulated miRNAs and monitor aneurysm progression and rupture. The latter would establish if the identified targets are involved in the crucial steps of aneurysm pathophysiology and delineate the mechanism behind cerebral aneurysm progression and rupture.

Third, there is a plethora of cells that are collected and analyzed together with the aneurysm tissue, although their concentration is not expected to be different among different samples. Fourth, our results are limited to aneurysms that were large enough to allow safe resection during surgery. The molecular profiles of the smaller aneurysms might be different, and our findings do not necessarily apply to that population. Fifth, we collected aneurysms for which treatment was deemed necessary, either due to patient preference or because the aneurysm harbored some dangerous features. Though these concerns limit the generalization of our results, acquiring tissue from aneurysms that are deemed appropriate for observation is not feasible. Sixth, our sample size is limited. However, this is a preliminary single-center study, which can be used as the basis for further multicenter investigations.

Conclusions

Although there is still much work to be done to fully understand the molecular mechanisms behind cerebral aneurysm formation and rupture, we have uncovered some likely facets of gene regulation in these tissues. Our analysis identified several significantly differentially expressed genes and miRNAs in unruptured human cerebral aneurysms. The strongest changes in expression were observed for miR-21, miR-143, miR-145, and their target genes. The majority of these genes are involved in collagen formation, inflammation regulation, lipid metabolism, smooth muscle phenotypic modification, and ECM remodeling, processes which have been implicated in cerebral aneurysm formation. Further research to separate causative and responsive gene expression differences may identify potential therapeutic targets among these miRNA-mRNA pairs.

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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: Bekelis. Acquisition of data: Bekelis, Kerley-Hamilton, Teegarden, Tomlinson, Kuintzle, Simmons, Singer, Roberts, Hendrix. Analysis and interpretation of data: Bekelis, Kerley-Hamilton, Teegarden, Tomlinson, Kuintzle, Kellis, Hendrix. Drafting the article: Bekelis. Critically revising the article: all authors. Reviewed submitted version of manuscript: Bekelis, Simmons, Hendrix. Approved the final version of the manuscript on behalf of all authors: Bekelis. Statistical analysis: Teegarden, Kuintzle, Kellis, Hendrix. Administrative/technical/material support: Bekelis, Kerley-Hamilton, Tomlinson, Singer, Kellis, Hendrix.

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