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ORIGINAL ARTICLE

Placenta microRNA profile of patient with Obstetric Antiphospholipid Syndrome

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Abstract

The onset of obstetric antiphospholipid syndrome (APS) occurs when antiphospholipid antibodies act upon the placenta. During pregnancy, APS exhibits traits such as vascular thrombosis, inflammation, and hindered trophoblast implantation. The involvement of microRNA expression has been proposed as a genetic factor contributing to the syndrome's development. MicroRNAs play a role in regulating gene expression in various cellular processes, including the formation of placental tissue. Therefore, additional research is needed to explore the control of placental miRNA in APS. In this study, we aimed to profile miRNA expressions from placenta tissue of patients with APS. Differentially expressed miRNAs were determined for its targeted genes and pathways. Agilent microarray platform was used to measure placental microRNA expressions between normal placental tissue and those obtained from patients with APS. Differentially expressed miRNAs were detected using GeneSpring GX software 14.2 and sequences were mapped using TargetScan software to generate the predicted target genes. Pathway analysis for the genes was then performed on PANTHER and REACTOME software. Selected miRNAs and their associated genes of interest were validated using qPCR. Microarray findings revealed, 9 downregulated and 21 upregulated miRNAs expressed in placenta of patients with APS. Quantitative expressions of 3 selected miRNAs were in agreement with the microarray findings, however only miR-525-5p expression was statistically significant. Pathway analysis revealed that the targeted genes of differentially expressed miRNAs were involved in several hypothesised signalling pathways such as the vascular endothelial (VE) growth factor (VEGF) and inflammatory pathways. VE-cadherin, ras homolog member A (RHOA) and tyrosine kinase receptor (KIT) showed significant downregulation while Retinoblastoma gene (RET), Dual specificity protein phosphatase 10 (DUSP10) and B-lymphocyte kinase (BLK) genes were significantly upregulated. These preliminary findings suggest the involvement of miRNAs and identified novel associated genes involvement in the mechanism of obstetric APS, particularly through the alteration of vascular-associated regulators and the inflammatory signalling cascade.

Keywords: MicroRNA, microarray, gene expression, placenta, Obstetric Antiphospholipid Syndrome, bioinformatics

INTRODUCTION

Antiphospholipid Syndrome (APS) is characterised by the presence of antiphospholipid antibodies and one or more thrombotic or pregnancy-related clinical features.[1] Diagnosing obstetric APS in suspected patients requires multiple tests at different time points to improve accuracy using current serologic methods. Early and accurate diagnosis has led to successful pregnancies in around 70% of obstetric APS cases, as it enables prompt initiation of appropriate treatment.[2] However, even with careful treatment and medication, approximately 30% of obstetric APS patients remain at risk of unsuccessful pregnancies[2], suggesting the

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existence of other mechanisms contributing to obstetric APS. Furthermore, some studies have shown devastating clinical manifestations of APS despite consistently negative laboratory results, emphasising the need to review the diagnostic strategy for APS.[3]

Earlier research associated obstetric APS with placental infarction or placental thrombosis[4], indicating the importance of the placenta in its pathogenesis. A relevant study in this context is related to preeclampsia, where a regulatory molecule called microRNA (miRNA) was found to play a role in its pathogenesis.[5] miRNAs are short non-coding RNAs consisting of 22 nucleotides that can interfere with post-transcriptional gene regulation. They can inhibit mRNA translation and promote mRNA degradation. Around 30% of genes are known to be regulated by miRNAs[6], and one gene can be targeted by multiple miRNAs.[7] This opens up an intriguing avenue for investigating the interaction between miRNA and gene expression networks in the underlying molecular mechanisms of obstetric APS.

Recent studies have highlighted the essential role of miRNAs in regulating placental development, influencing processes such as cell differentiation, apoptosis, inflammation, and angiogenesis.[8] Abnormal miRNA expression has been associated with pregnancy complications and APS, and miRNA has been shown to affect the endothelium in recurrent miscarriages.[9] Aberrant miRNA expression in disease conditions can serve as an indicator of diseases, potential biomarkers, and therapeutic targets.[10] Given the involvement of miRNAs in various placenta-related disease mechanisms, this study aimed to explore miRNA expression in placental tissue from patients with obstetric APS. Additionally, further analysis was conducted to identify potential biomarkers and signalling pathways associated with obstetric APS based on the targeted genes of these miRNAs.

METHODOLOGY

Tissue Samples

This comparative cross-sectional study involved patients who were diagnosed with obstetric APS. A comprehensive set of inclusion and exclusion criteria was developed to recruit samples for the APS and normal control groups, following the Sydney criteria (Table 1). The collected samples comprised whole blood, collected in citrated tubes (BD Vacutainer, USA), for lupus anticoagulant testing, and placental tissue immersed in 3-5 ml of RNA later (Ambion™, USA) for RNA extraction. All samples were obtained from consenting patients who had experienced a miscarriage, preeclampsia, or intrauterine death.

MicroRNA Microarray for Target Gene and Pathway Analysis

Total RNA was extracted following the manufacturer's recommendations (Ambion™, USA). The RNA pellet was then purified, and the RNA Integrity Number (RIN) was measured using the Agilent 2100 Bioanalyzer (Agilent, USA). Samples with a reading of approximately ~2.0 for both A260/280 and A260/230 were selected for further analysis. To ensure high-quality RNA, a minimum RIN threshold of ~7.0 was set to eliminate potential experimental bias caused by poor RNA quality prior to microarray profiling analysis and quantitative polymerase chain reaction (qPCR).

For miRNA microarray profiling, the G3 Human miRNA Microarray, Release 21, 8x60k slide (Agilent, USA) was used, following the manufacturer's guidelines. It contained 2549 human probes targeted against human microRNA sequences from miRBase 21.0. The process involved the preparation of spike-in solutions, dephosphorylation, denaturation, and ligation of the samples, followed by drying, hybridisation, washing, and slide scanning.

The microarray-generated scan image was analysed using Feature Extraction Software Version 9.5.3 to extract probe features. The data obtained from the scanned images were analysed using Gene Spring GX 14.2 software to obtain the expression value and identify differentially expressed miRNAs. The list of differentially expressed miRNAs was filtered using the TargetScan program to obtain the target genes of the miRNAs. A fold change of ≥ 2 and $p < 0.05$ (determined by modified T-test) was set as the threshold to filter out predicted target genes. The set of genes that remained after the fold change and p-value filtering were further analysed using PANTHER[11] and Reactome program[12] to identify the pathways associated with antiphospholipid syndrome. Selected genes from the pathway of interest were then validated using qPCR.

Validation of microRNA Expression by qPCR

For cDNA synthesis of microRNA, the manufacturer's protocol was followed (Qiagen, USA). Primers for human hsa-miR-3148, hsa-miR-670, hsa-miR-525, RNU6-2, and SNORD48

TABLE 1: Inclusion and exclusion criteria for sample selection

Normal group	
Inclusion criteria	**Exclusion criteria**
• Normal spontaneous vaginal delivery or through caesarean procedure. • Does not have pregnancy loss history. • Age ranging from 18-40 years' old • Negative for all LA-aPTT, dRVVT and anti-β2GP1 test	• Intrauterine infectious diseases such as Toxoplasmosis, Other infections (Coxsackievirus, Chicken Pox, Parvovirus B19, Chlamydia, HIV, Syphilis, Human T-lymphotrophic Virus), Rubella, Cytomegalovirus and Herpes Simplex virus-2 or • Gestational Diabetes Mellitus, or • Cervical Incompetence, or • Related Autoimmune Disease such as Systemic Lupus Erythematosus. • Positive for LA-aPTT and/or dRVVT and/ or anti-β2GP1 test
APS group	
Inclusion criteria	**Exclusion criteria**
• Successful birth where mother is already diagnosed with aPLs antibodies • History of recurrent miscarriages (≥3) at ≥16 weeks' period of gestation. • History of 2 miscarriages, current miscarriage at ≥ 16 weeks' period of gestation. • Complication of intrauterine death • Delivery with preeclampsia or severe eclampsia at any gestation period. • Intrauterine Growth Restriction at any gestation period. • Age ranging from 20 to 40 years' old • Positive for LA-aPTT and/or dRVVT and/ or anti-β2GP1 test	• Intrauterine infectious diseases such as Toxoplasmosis, Other infections (Coxsackievirus, Chicken Pox, Parvovirus B19, Chlamydia, HIV, Syphilis, Human T-lymphotrophic Virus), Rubella, Cytomegalovirus and Herpes Simplex virus-2 or • Gestational Diabetes Mellitus, or • Cervical Incompetence, or • Related Autoimmune Disease such as Systemic Lupus Erythematosus. • Negative for all LA-aPTT, dRVVT and anti-β2GP1 test

Abbreviation: LA-aPTT; lupus antigoaculant-activated partial thromboplastin time, dRVVT; dilute Russell's viper venom and anti-β2GP1; anti-β2-Glycoprotein1

were purchased from Qiagen, USA, for miRNA quantification. Quantitative PCR (qPCR) for miRNA was performed using the StepOnePlus Real-Time PCR System (Applied Biosystem, USA).

miRNA Target-Gene Expression Analysis by qPCR

For the cDNA synthesis of targeted genes, the Superscript III First-Strand Synthesis SuperMix (Invitrogen, USA) was utilised. The primer sequences were designed using the Primer3 input tool (http://primer3.ut.ee) based on the coding region sequences of each gene, as referenced from the GeneBank data. The newly designed primers for the differentially expressed miRNA targeted-genes are listed in Table 2. Five genes, namely, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Platelet and endothelial cell adhesion molecule-1 (PECAM-1), Vascular endothelial cadherin (VE-cadherin), Endothelial Nitric Oxide Synthase (eNOS), and Von Willebrand factor (vWF), were selected from angiogenic-endogenic-associated genes studied by Nur Fariha *et al*. in 2012. These four genes were chosen based on their association with the pathway analysis of upregulated miRNAs-targeted genes.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism Software v5.0. The qPCR

TABLE 2: Primer sequences for upregulated miRNA targeted-genes

Gene	Accession number	Primer Sequences (5`→3`) Sense and antisense	Size, base pair (bp)
Upregulated miRNA target-genes			
Ras homolog family member A (RHOA) Transcript variant 2	NM_001313941.1	GTGGCAGATATCGAGGTGGA ACTATCAGGGCTGTCGATGG	147
Homo sapiens vascular endothelial growth factor D (VEGFD) FIGF (VEGFD)	NM_004469.4	GTGCAGCCCTAGAGAAACGT GTACGAGGTGCTGGTGTTCA	151
IQGAP1 Homo sapiens IQ motif containing GTPase activating protein 1 (IQGAP1)	NM_003870.3	TGCTGAAGGACTCGTTGCAT AGATTTCGGCGTTGGTCTGT	178
NCAM1 Neural cell adhesion molecule 1 (NCAM1), transcript variant 2, mRNA	NM_181351.4	GGAGAGGACCCCAAACCATG TCGTTTCTGTCTCCTGGCAC	119
KIT KIT proto-oncogene receptor tyrosine kinase (KIT), transcript variant 1	NM_000222.2	TCGGCTCTGTCTGCATTGTT ACACAGACACAACAGGCACA	110
RET Homo sapiens ret proto-oncogene (RET), transcript variant 4	NM_020630.4	AAGCTGTATGTGGACCAGGC TATGGTCCAGGCTCCGGTTA	196
FGA fibrinogen alpha chain (FGA), transcript variant alpha	NM_021871.3 NM_000508.4	CTGCCTGGTCCTAAGTGTGG GCAGAAGGGCCAGTCTGAAT	145
RPS27A ribosomal protein S27a (RPS27A), transcript variant 3	NM_001177413.1	AGGTTAAGCTGGCTGTCCTG TTGCCATAAACACCCCAGCA	111
MAPK12 mitogen-activated protein kinase 12 (MAPK12), transcript variant 1	NM_002969.4	CTCTGGCTGTGAACCTCCTG ATACTTCTGGACCTGGGGCT	134
Homo sapiens dual specificity phosphatase 10 (DUSP10), transcript variant 1, mRNA DUSP10	NM_007207.5	CAACTCCCTCCAGCTCCAAG AACAGGAAGGGCAAGATGGG	135
protein tyrosine kinase 2 (PTK2), transcript variant 4	NM_001316342.1	AAACCAGATCCTGCAGCTCC GGCTTGACACCCTCGTTGTA	116

Downregulated miRNA target-genes			
B-Lymphocyte Kinase (BLK)	NM_001715.2	GGC CAT TAA GAC GCT GAA GG ATC CTC TGG CCA TGT ACT CG	158
Cluster of Differentiation (CD19)	NM_001178098.1	GAA AGC GAA TGA CTG ACC CC GCT GCT CGG GTT TCC ATA AG	182
Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 (MAPKAPK2)	NM_004759.4	ACC GTA CTA TGT GGC TCC AG ATG GTC ATT CTC TGG GTG GG	270
Myosin Heavy Chain 15 (MYH15)	NM_014981.1	GTA GAT GAC CTC CTG ACC CG CTT TTG TGC TGC CAG GTC AT	150

results are presented as mean ± standard deviation (SD). For the comparison of two groups, the Mann-Whitney test was employed, with a significance level of $p < 0.05$ considered as statistically significant to determine the differential expression of genes and miRNAs between APS and normal samples.

RESULT

Patient Profile

Placenta tissue and blood samples were collected from 5 normal cases and 3 cases of APS. The serological tests for APS were performed on all samples, and the placenta tissue was processed for microRNA microarray analysis. In the normal pregnancy group, all samples tested negative for lupus anticoagulant (LA), anticardiolipin antibodies (aCL), and anti-β2-glycoprotein1 (anti-β2GP1) antibodies. The table below (Table 3) provides detailed information on pregnancy complications and serological test results for all APS cases.

MicroRNA microarray profile

Volcano plot, Principal component analysis and Hierarchial clustering of samples

By applying a corrected p-value cut-off of 0.05 ($p < 0.05$) and a fold change cut-off of 2.0 (>2.0), we identified 30 miRNAs that were differentially expressed: 21 were upregulated and 9 were downregulated, as depicted in Figure 1 (A). In the figure, highly upregulated miRNAs are positioned towards the right side, while highly downregulated miRNAs are positioned towards the left side. The upregulated miRNAs with statistical significance below 0.05 are marked in red, while the downregulated miRNAs with statistical significance below 0.05 are marked in blue. MiRNAs with fold changes less than 2 and lacking statistical significance are shown in grey. The differentially expressed miRNAs are listed in Table 4.

To explore the sample arrangement between the normal and APS groups, an exploratory principal component analysis (PCA) was conducted using the analysis platform embedded in the GeneSpring GX 14.5 Software. The PCA plot in Figure 1 (B) clearly demonstrates the distinct clustering of samples between the normal and APS groups.

Furthermore, hierarchical clustering was performed on the one-channel microarray data to assess the similarity or differences in miRNA profiles among patient samples. The dendrogram in Figure 2 represents the comparison between normal and APS samples. The heatmap reveals that technical and biological replicates from the same group of samples are grouped together, providing insights into the similarity among the miRNA profiles.

TABLE 3: Serological profile of APS patients

Sample	Complications / Period of gestation	Tests		
		LA	aCL	anti-β2GP1
APS 1	Early miscarriage / 19 weeks	Positive	Positive	Negative
APS 2	PE, miscarriage / 26 weeks	Positive	Positive	Positive
APS 3	Early miscarriage / 8 weeks	Positive	Positive	Negative

Abbreviation: PE: Pre-eclampsia; aCL; anti-cardiolipin

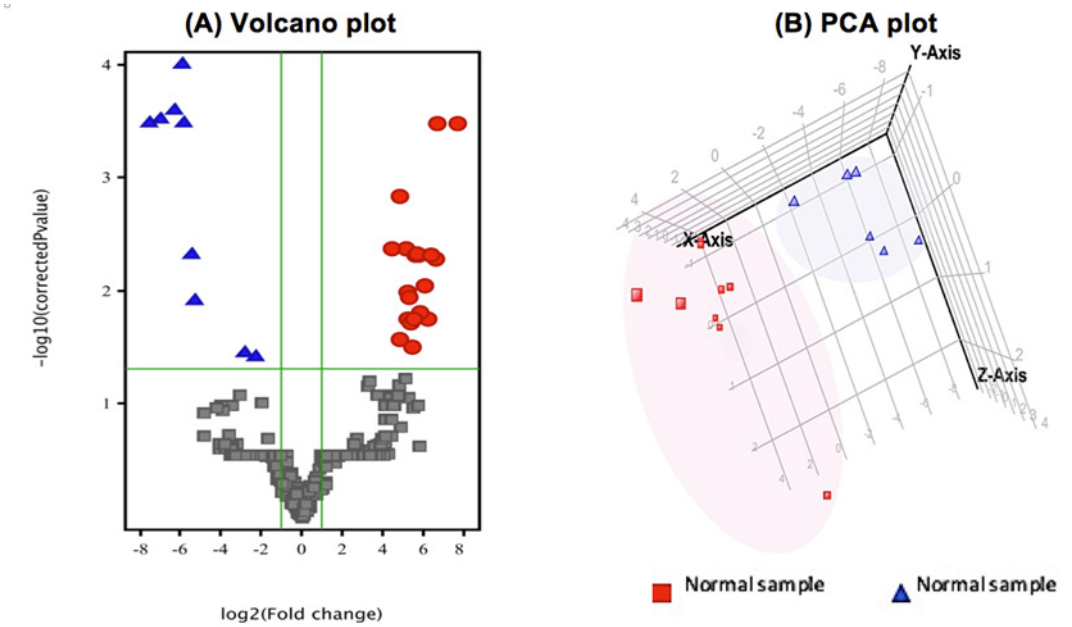

FIG. 1: Volcano and PCA plot for comparison between APS and normal samples. Volcano (A) and PCA (B) plots for APS versus normal samples. For the volcano plot, up- and downregulated miRNA were indicated in red and blue respectively. whereas downregulated miRNA. miRNAs which appear in grey were not statistically significant in expression.

MicroRNA-targeted genes and pathways

Using the TargetScan computational algorithm to compare normal and APS samples, it was predicted that the upregulated miRNAs would target 578 genes, while the downregulated miRNAs would target 178 genes. To gain insights

TABLE 4: Differentially expressed miRNAs in normal versus APS samples

Upregulated miRNAs	Downregulated miRNAs
hsa-miR-1238-5p	hsa-miR-1183
hsa-miR-1825	hsa-miR-1914-3p
hsa-miR-2278	hsa-miR-4497
hsa-miR-3148	hsa-miR-4689
hsa-miR-3907	hsa-miR-518e-5p
hsa-miR-3912-5p	hsa-miR-525-5p
hsa-miR-4436b-5p	hsa-miR-564
hsa-miR-4502	hsa-miR-6768-5p
hsa-miR-4644	
hsa-miR-4730	
hsa-miR-4769-3p	
hsa-miR-5007-5p	
hsa-miR-595	
hsa-miR-670-5p	
hsa-miR-6739-5p	
hsa-miR-6751-3p	
hsa-miR-6751-5p	
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hsa-miR-7844-5p	
hsa-miR-7850-5p	

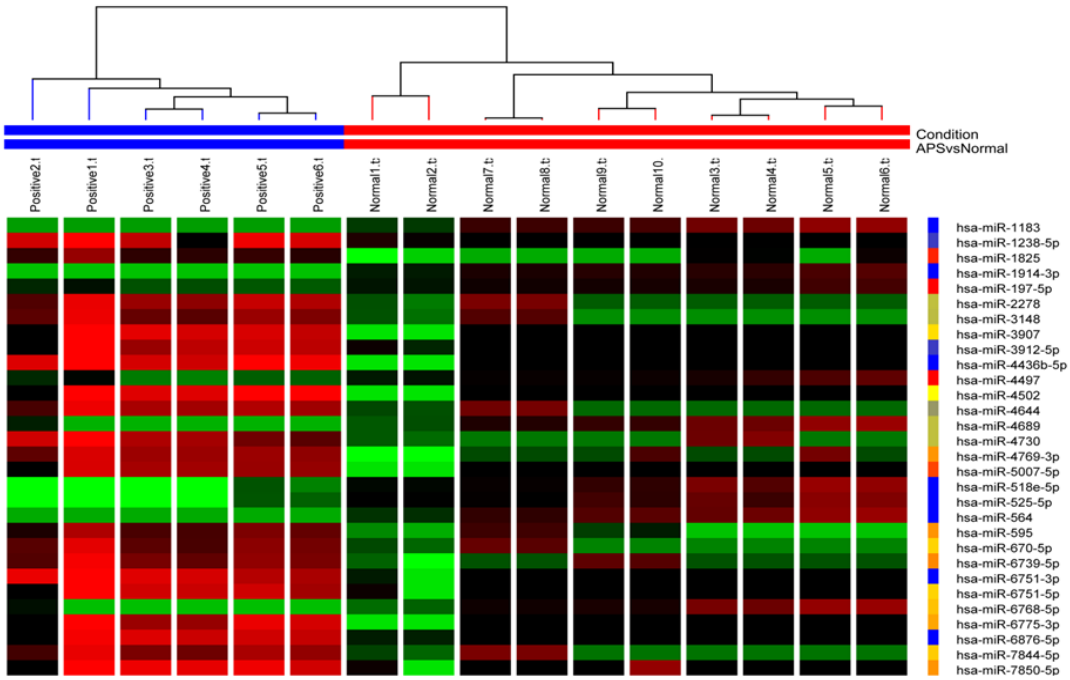

FIG. 2: Hierarchical clustering of normal versus APS samples. The colours represent the expression levels of the miRNAs. Red represents high expression while green represents low expression. The right ledger shows the differentially expressed miRNAs and top ledger shows the samples.

into the biological processes associated with these genes, gene ontology (GO) analysis was performed using DAVID, focusing specifically on the Biological Process (BP) GO term. The top 10 BP GO terms are listed in Table 5 for the upregulated miRNA-targeted genes and in Table 6 for the downregulated miRNA-targeted genes.

Moreover, pathway analysis of the upregulated miRNAs revealed 1168 pathways, with a particular emphasis on the VEGF-VEGFR pathway due to its relevance to the altered vascular functions in obstetric APS. On the other hand, the downregulated miRNA-targeted genes were found to be involved in 71 pathways, with a focus on pathways related to inflammatory processes. These pathways include B-cell activation, interleukin signalling pathway, inflammation mediated by chemokine and cytokine signalling pathways, and toll receptor signalling pathways. Table 7 lists several genes selected from these pathways for qPCR analysis.

TABLE 5: GO analysis on biological process of upregulated miRNAs-targeted genes

GO Term	Gene count	p-value
Endoplasmic reticulum mannose trimming	11	6.6×10^{-3}
G2/M transition of mitotic cell cycle	4	1.2×10^{-2}
Response to fatty acid	4	1.4×10^{-2}
Response to vitamin A	5	1.5×10^{-2}
Glucose transport	4	1.7×10^{-2}
Negative regulation of endoplasmic reticulum stress-induced intrinsic apoptotic signaling pathway	4	1.9×10^{-2}
Flavonoid biosynthetic process	4	2.5×10^{-2}
Flavonoid glucuronidation	11	2.5×10^{-2}
Metabolic process	3	3.2×10^{-2}

TABLE 6: GO analysis on biological process of downregulated miRNAs-targeted genes

GO Term	Gene Count	p-value
Response to nutrient	15	6.0×10^{-3}
Notch signalling pathway	14	9.3×10^{-3}
Optic nerve development	4	9.4×10^{-3}
G2/M transition of mitotic cell cycle	14	2.4×10^{-2}
Carboxylic acid metabolic process	44	2.4×10^{-2}
Oxoacid metabolic process	44	2.6×10^{-2}
Positive regulation of sperm motility	3	2.7×10^{-2}
Atrioventricular valve formation	3	2.7×10^{-2}
Kidney development	18	2.8×10^{-2}
Response to vitamin A	4	2.9×10^{-2}

Quantitative expression of miRNAs and genes

We performed quantification of the upregulated miRNAs miR-670 and miR-3148 in APS compared to normal samples, which showed a 3-fold and 4-fold change, respectively. However, these differentially expressed miRNAs did not reach statistical significance ($p < 0.05$, Mann-Whitney U-test). On the other hand, we quantified the downregulated miRNA miR-525-5p using qPCR, and the results revealed a significant downregulation of miR-525-5p in the APS group compared to the normal group. Detailed results can be found in Table 8, and Figure 3 provides a graphical representation of the findings.

Quantitative expression of predicted miRNA-targeted genes

Based on the qPCR data, we observed that most of the genes (PECAM-1, vWF, Ve-cadherin, RHOA, IQGAP, NCAM1, KIT, FGA, RPS27A, and PTK2) were upregulated in APS tissue compared to normal tissue. Additionally, three genes (RET, MAPK12, and DUSP10) were downregulated. However, the expression of eNOS showed no significant difference between APS and normal samples (Figure 4).

Further analysis revealed that out of the 13 genes quantified, only five genes (VE-cadherin, RHOA, KIT, RET, and DUSP10) showed a significant difference between APS and normal samples ($p < 0.05$, Mann-Whitney U test). These genes may play a crucial role in the pathogenesis of obstetric APS.

Moreover, Figure 5 demonstrates that among the four genes quantified by qPCR, only BLK showed a significant upregulation between the APS and normal groups. This suggests that BLK may have a potential role in the development of obstetric APS.

DISCUSSION

The concept of miRNA-regulated mechanisms in disease has been recognised for some time, but the involvement of these molecules in APS (antiphospholipid syndrome) has remained largely unknown. Previous studies have documented the role of miRNAs in various molecular mechanisms underlying diseases.[13] For example, the downregulation of miR-19b and miR-20a, members of the miR-17~92 cluster, was inversely correlated with tissue factor (TF) expression. TF is a primary initiator of blood coagulation, and increased TF expression can trigger a hypercoagulable state.[14] These studies have primarily focused on known genes expressed in specific cell types and miRNAs that play key roles in those cells. In our study, miRNAs were selected using an in-silico method, which predicts miRNAs based on web databases and algorithms. This selection was based on the genes of interest uploaded to the database. Microarray analysis revealed unique miRNA expression patterns in APS compared to normal samples, as evidenced by clear separation between the APS and normal groups in PCA plot and hierarchical clustering.

We identified 30 differentially expressed miRNAs in APS, with 21 miRNAs significantly upregulated and 9 miRNAs downregulated. Among the upregulated miRNAs, miR-3148 targeted the highest number of genes (12 genes in total). Previous research on miR-3148 in systemic lupus erythematosus (SLE), an autoimmune disease, showed that its overexpression correlated with downregulated mRNA expression of Toll-like receptor 7.[15] Additionally, miR-670-5p, miR-4644, and miR-595 were found to target 7 genes each. miR-670-5p is associated with cell

TABLE 7: Selected genes from pathways of interest

miRNA regulation detected by microarray	miRNA	miRNAs target gene	Pathway
Upregulated miRNAs	miR-2278, miR-4644, miR-4769-3p, miR-3148	ras homolog family member A (RHOA)	VEGFA-VEGFR
	miR-670-5p, miR-3907, miR-4436b-5p, miR-4769-3p	IQ motif containing GTPase activating protein 1 (IQGAP1)	VEGFA-VEGFR
	miR-670-5p, miR-1825, miR-595, miR-4644, miR-4769-3p, miR-3148	neural cell adhesion molecule 1 (NCAM1)	VEGFA-VEGFR
	miR-1825, miR-595, miR-4502, miR-2278, miR-4769-3p	KIT proto-oncogene receptor tyrosine kinase (KIT)	VEGFA-VEGFR
	miR-1825, miR-670-5p, miR-2278, miR-4436b-5p, miR-4769-3p, miR-4730	ret proto-oncogene (RET)	VEGFA-VEGFR
	miR-595, miR-4436b-5p, miR-4769-3p, miR-3148	fibrinogen alpha chain (FGA)	VEGFA-VEGFR
	miR-3907, miR-4769-3p	ribosomal protein S27a (RPS27A)	VEGFA-VEGFR
	miR-670-5p, miR-2278, miR-3907	mitogen-activated protein kinase 12 (MAPK12)	VEGFA-VEGFR
	miR-595, miR-2278, miR-4644, miR-3148	dual specificity phosphatase 10 (DUSP10)	VEGFA-VEGFR
	miR-1825, miR-595, miR-3907, miR-3148	protein tyrosine kinase 2 (PTK2)	VEGFA-VEGFR
Downregulated miRNAs	miR-564, miR-4497	B-lymphocyte Kinase (BLK)	B-cell activation
	miR-525-5p, miR-4689	Cluster of Differentiation 19 (CD19)	B-cell activation
	miR-525-5p, miR-564, miR-4689	Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 (MAPKAPK2)	Interleukin signalling pathway
	miR-1183, miR-4497, miR-4689	Myosin Heavy Chain 15 (MYH15)	Inflammation mediated by chemokine and cytokine signalling pathway

proliferation and targets Prospero Homeobox 1 (PROX1), which plays a role in vessel remodelling.[16] The downregulation of PROX1 could disrupt its role in vessel remodelling and contribute to vascular thrombosis in APS placenta. miR-595 has been implicated in inflammatory bowel disease and may be involved in the inflammatory-mediated tissue damage observed in APS placenta.[19] miR-4644 has been identified as a potential diagnostic marker for pancreatic cancer but has not been linked to other diseases in the literature.[20]

Interestingly, the differentially expressed miRNAs in APS identified in our study

TABLE 8: Expression pattern of selected miRNAs from qPCR data

Systematic name	P value	Regulation *APS compare to normal	Fold change
miR-670	0.5167	UP	3.94
miR-3148	0.4121	UP	4.99
miR-525-5p	0.0121	DOWN	232.14

were distinct from those found in non-APS placenta samples from early pregnancy loss and preeclampsia.[5,21-22] When we examined the predicted target genes of the upregulated miRNAs using the REACTOME platform, we found that these genes were associated with the VEGF-VEGFR pathway, which includes vascular-associated genes involved in angiogenesis. Inhibition of angiogenic factor secretion and impairment of placental angiogenesis have been suggested as causes of aPL-mediated foetal loss.[24] The effects of aPLs on trophoblasts include limiting trophoblast migration, suppressing placenta growth factor production, and modulating trophoblast angiogenic factor secretion.[25-27]

The qPCR findings of genes in VEGF-VEGFR pathway supported our hypothesis that the targeted genes were predicted to be downregulated due to suppression by the upregulated miRNAs. However, it is worth noting there were targeted genes that were either upregulated or unchanged in its expression. VE-cadherin, RHOA and KIT were significantly downregulated while RET and DUSP10 were significantly upregulated (Figure 4). VE-cadherin has been found to enhance cytotrophoblast invasiveness.[28] Damsky *et al*. reported that

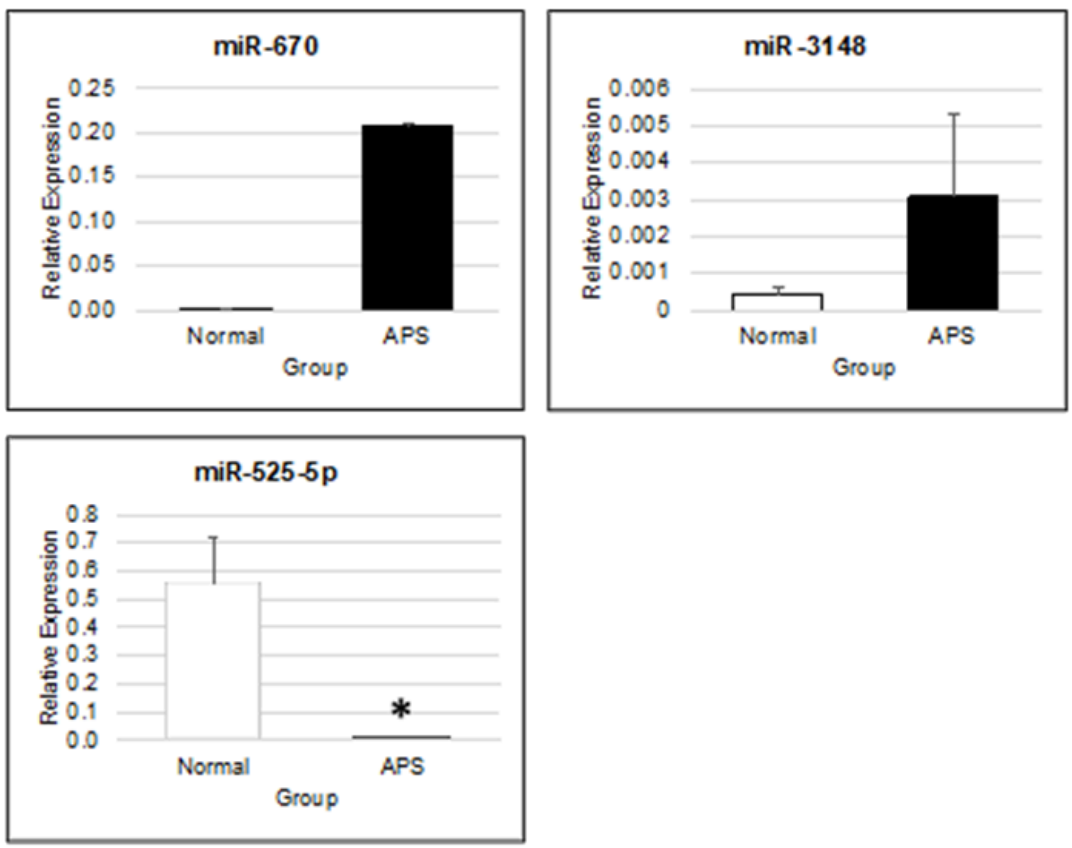

FIG. 3: Relative expression of miR-670, miR-3148 and miR-525-5p. Relative expression (mean ± SEM) of miR-670, miR-3148 and miR-525-5p in normal placenta (Normal, n = 4) and placenta of patient with APS (APS, n = 2), determined by real-time PCR. (* indicate $p < 0.05$)

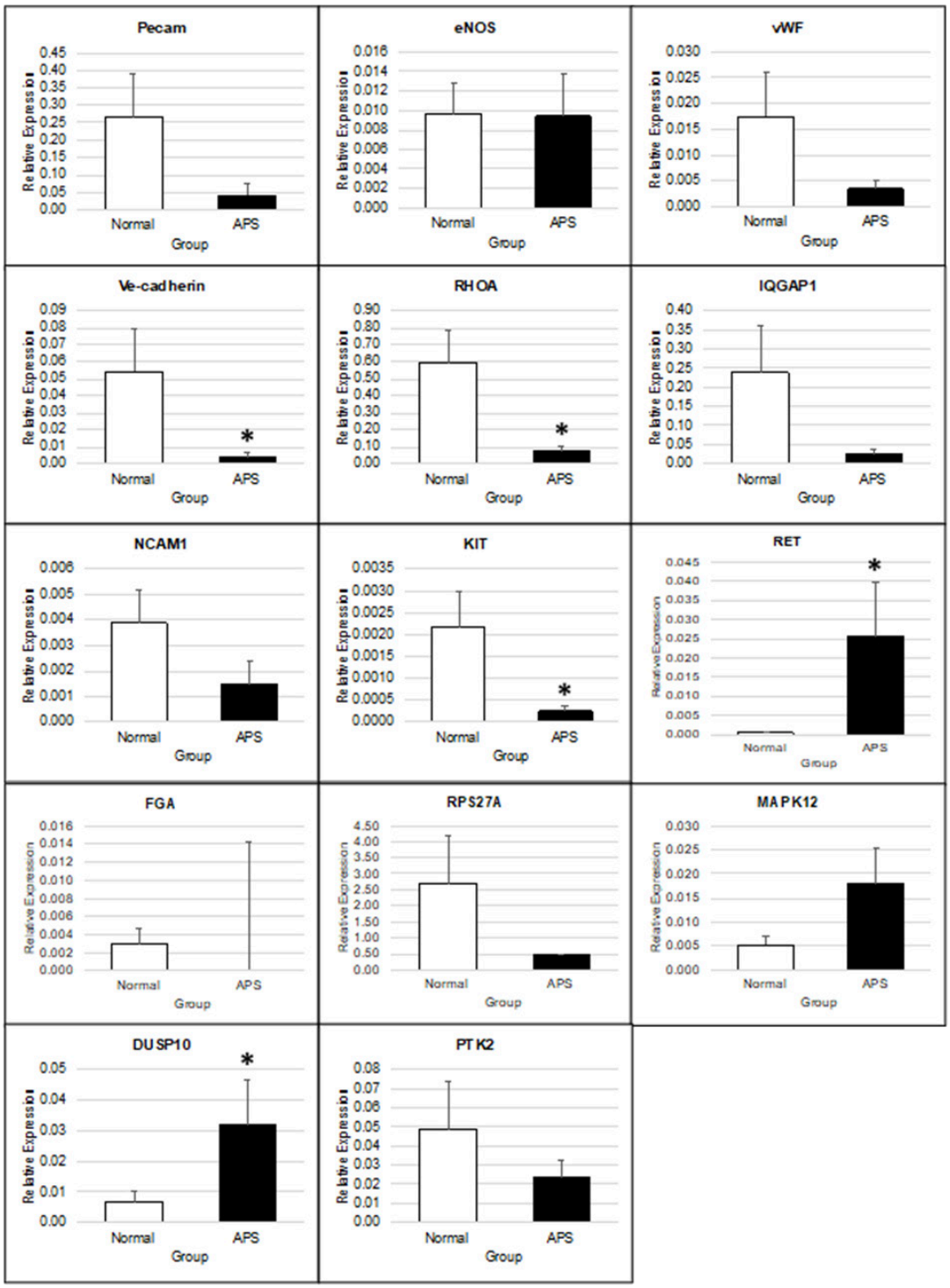

FIG. 4: Relative expression of upregulated miRNA-targeted genes. Relative expression (mean ± SEM) of up-regulated miRNA-targeted genes and vascular-associated genes in placentas of patient with APS (APS, n = 2) and normal (Normal, n = 4), determined by real-time PCR. (* indicate $p < 0.05$)

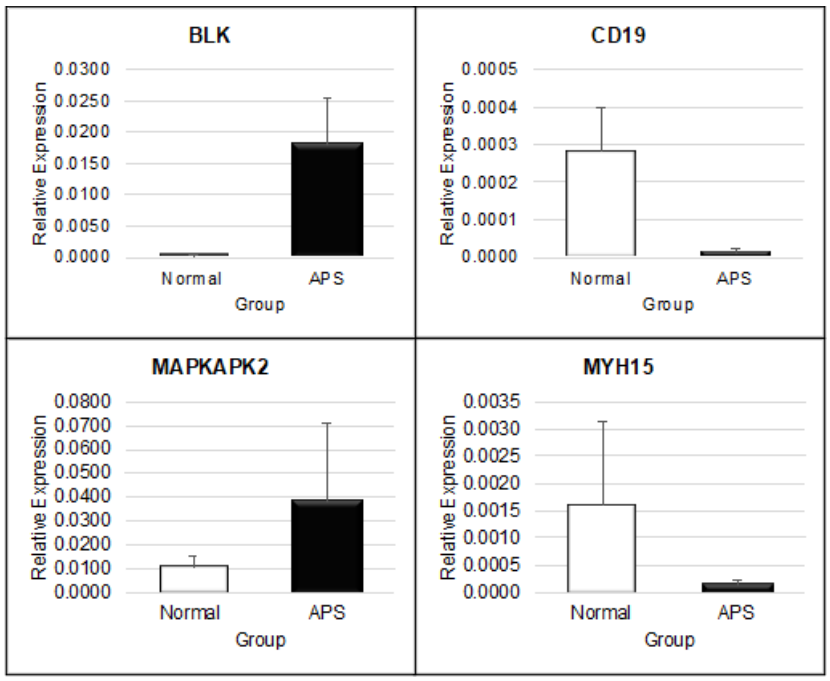

FIG. 5: **Relative expression of downregulated miRNA-targeted genes.** Relative expression (mean ± SEM) of downregulated miRNA-targeted genes in placentas from obstetric APS (APS, n = 2) and normal (Normal, n = 4), determined by real-time PCR. (* indicate $p<0.05$)

antiphospholipid antibodies caused the down regulation of VE-cadherin, hence causing impaired trophoblast invasion.[29] Meanwhile, RHOA, which is known as a modulator of gene expression, adhesion and migration of activated macrophage, plays an important role in NFκB inflammatory signalling.[30] RHOA protein was found to be significantly increased in monocytes of APS patients with thrombosis but decreased as compared with monocytes from APS without thrombosis.[31] This may suggest that the downregulation and upregulation of RHOA may be directly associated to the thrombotic state. Significant downregulation of RHOA in our study may indicate the association of this marker to mechanisms of APS other than thrombosis. The other 3 genes have never been reported in any APS study. However, the role of KIT was stressed in the development of placenta tissue in-utero. mRNA expression of KIT detected at the stages of preimplantation and placenta development is suggestive of its role in promoting proliferation and differentiation of the placenta cells.[32] Suppression of KIT in our findings may indicate placental insufficiency which could be one of the pathogenic mechanisms of APS.

From the results of RET and DUSP10 qPCR quantification, the expressions were 3 times higher in APS compared to normal sample. DUSP10 functions by inactivating their target kinases through dephosphorylating both phosphoserine/threonine and phosphotyrosine residue. DUSP10 negatively regulate members of MAP kinase superfamily which are associated with cellular proliferation and differentiation. The upregulated DUSP10 might have an association to downregulated level of KIT as DUSP10 functions to reduce activated tyrosine kinases which further results in desensitization of the kinase receptor. RET is a member of the cadherin superfamily, which is encoded for receptor tyrosine kinases, a cell-surface molecule that transduces signal for cell growth and differentiation.[33] RET mutation was previously reported to increase angiogenesis in thyroid carcinoma.[34,35] APS placenta was reported to have poor blood circulation due to infarction.[36] In response to infarction, APS placenta might have increased vascular formation as a compensation mechanism. Our results on RET expression may reflect the hypothesis where RET may act as an indicator of increased angiogenesis. A study by Caroll *et al*. found that the treatment of aPLs on trophoblast cells altered the trophoblasts' angiogenic factors, where the anti-β2GPI-exposed trophoblast produced higher VEGF and PIGF.[27]

miR-525-5p was validated by qPCR and

showed significant downregulation in APS sample with large fold changes (Table 17). miR-525-5p was predicted to target CD19 and MAPKAPK2. However, only MAPKAPK2 expression was upregulated and in correlation to the reduced expressions of miR-525-5p but the data was not significant. CD19 may not be the true target of miR-525-5p or it is also a target of other upregulated miRNAs, as one gene can be controlled by more than one miRNA and one miRNA can regulate multiple genes. Hromadnikova *et al*. reported that the downregulation of some C19MC miRNAs including miR-525-5p was found to be a common phenomenon shared between placenta tissue from gestational hypertension, preeclampsia and foetal growth restriction. Further analysis on predicted miR-525-5p's target genes using miRWalk database revealed other genes than CD19 and MAPKAPK2.[37]

By using pathway analysis, BLK was identified as the downregulated miRNA-targeted gene (Figure 5). BLK is known to be involved in B-cell activation. BLK is targeted by other miRNAs which were not validated by qPCR in this study, miR-564 and miRNA-4497. Therefore, we could not make a correlation between miRNAs related to BLK and their regulations on BLK in Obstetric APS. BLK gene encodes for a tyrosine kinase. This tyrosine kinase is involved in the regulation of B-cell activation and may influence the proliferation and differentiation of B-cells.[38] Studies showed that BLK expression is associated with primary APS[39] and SLE.[40,41] Presence of SLE in antiphospholipid syndrome is categorised as secondary APS. In addition, association of BLK with other rheumatic diseases such as rheumatoid arthritis (RA) and multiple sclerosis[41] has also been reported. This suggests that BLK could be the vulnerable gene in Obstetric APS. Hence, regulation of BLK by miRNAs should be further investigated. Another downregulated miRNA-targeted gene, MYH15, has never been reported to be involved in antiphospholipid syndrome or other rheumatic diseases. Recent study has suggested the role of MYH15 in Amyotrophic lateral sclerosis (ALS), a fatal neurological disorder characterised by progressive muscular atrophy and respiratory failure.[42] The reduced expression of MYH15 in APS sample by qPCR was also not in concordant to its downregulated miRNAs. However, MYH15 is involved in inflammatory signalling cascade and further investigation could clarify its regulation in APS.

This study therefore indicates the useful strategy of miRNA to narrow down the cohort of candidate transcriptomes that can be mapped to either biological processes or on the genes from pathways of interest in relation to the cell type or disease under investigation. Most notably, this is the first study to our knowledge, to investigate the profiling of miRNA from patient's tissue sample in obstetric APS. Our results provide compelling evidence for the involvement of certain genes to explain the molecular mechanism of APS. However, some limitations are worth noting. Although our hypotheses were supported statistically, the various experimental settings, normalisation application and platforms that may result in inconsistent outcomes of the differential expression of miRNA in the placenta tissues required strict monitoring. However, we have kept these variables to a minimum by adhering to protocols, optimising each step before actual test samples are run and running duplicates for each test run. The inclusion criteria could have also been made to be more homogeneous in terms of clinical criteria or gestational week among pregnant women of APS. Future work should therefore include follow-up work designed to evaluate these preliminary finding at a larger scale. Future multicentre studies should be carried out to obtain larger sample size which best reflect APS in obstetrics. Further work should also include the characterisation of significantly upregulated and downregulated genes identified in the current work at the protein level using RNA interference strategies.

In conclusion, we have identified the genetic expression of VE-cadherin, RHOA, RET, KIT and DUSP10 in VEGFA-VEGFR signalling pathway in placenta tissue of obstetric APS patients as compared to normal placenta. This finding suggests the inducing effect of antiphospholipid antibodies on miRNAs which control the regulation of genes in vascular-associated pathways. The data also demonstrated that not only miRNAs expressions were induced but some miRNAs were supressed. Inhibition of certain miRNAs may cause an overexpression of its targeted genes. It would be interesting to further characterise other miRNAs and its targeted genes to illustrate miRNA-associated mechanism of APS. miR-525-5p for example was greatly downregulated in APS thus, its associated genes would require further investigation by functional analysis. The relationship between miRNAs and its new target genes in placenta tissue can also be established as a novel database

on miRNA as the targeted genes are mainly based on cancer studies.

The qPCR findings in the VEGF-VEGFR pathway supported the hypothesis that the targeted genes would be downregulated due to suppression by the upregulated miRNAs. However, it is important to note that there were some targeted genes that showed either upregulation or no change in expression.

VE-cadherin, RHOA, and KIT were found to be significantly downregulated, while RET and DUSP10 were significantly upregulated. VE-cadherin has been shown to enhance cytotrophoblast invasiveness, and its downregulation may lead to impaired trophoblast invasion. RHOA, which plays a role in gene expression, adhesion, migration of activated macrophages, and NFκB inflammatory signalling, was significantly decreased in APS patients with thrombosis but increased compared to APS patients without thrombosis. This suggests that the downregulation of RHOA may be associated with mechanisms of APS other than thrombosis. KIT, on the other hand, is involved in the development of placenta tissue and its suppression may indicate placental insufficiency as a potential pathogenic mechanism of APS.

The significant upregulation of RET and DUSP10 in APS samples may have implications for angiogenesis. RET is known to be involved in cell growth and differentiation and has been linked to increased angiogenesis in thyroid carcinoma. In APS, poor blood circulation due to placental infarction has been reported, and the upregulation of RET may reflect a compensatory mechanism of increased vascular formation. DUSP10, which negatively regulates members of the MAP kinase superfamily associated with cellular proliferation and differentiation, may be associated with the downregulated level of KIT, as DUSP10 reduces activated tyrosine kinases, leading to receptor desensitisation.

miR-525-5p was found to be significantly downregulated in APS samples, and its target genes were predicted to include CD19 and MAPKAPK2. However, only MAPKAPK2 expression was upregulated, and the correlation between miR-525-5p and CD19 expression was not significant. It is possible that CD19 may not be the true target of miR-525-5p or that it is regulated by other upregulated miRNAs. Further analysis using miRWalk database revealed additional target genes for miR-525-5p.

Pathway analysis identified BLK as a downregulated miRNA-targeted gene. BLK is involved in B-cell activation and has been associated with primary APS, secondary APS (when APS coexists with systemic lupus erythematosus), rheumatoid arthritis, and multiple sclerosis. However, the correlation between miRNAs related to BLK and their regulation on BLK in Obstetric APS could not be established in this study.

MYH15, another downregulated miRNA-targeted gene, has not been previously reported in APS or other rheumatic diseases. Its role in inflammatory signalling cascade warrants further investigation to clarify its regulation in APS.
Overall, this study provides evidence for the involvement of specific genes in the molecular mechanism of APS in placental tissue. However, there are some limitations to consider, such as the need for strict monitoring of experimental settings, normalisation application, and platforms to minimise inconsistent outcomes. Future work should include larger-scale studies with homogeneous inclusion criteria and characterisation of identified genes at the protein level using RNA interference strategies.

In summary, the study revealed differential genetic expression of VE-cadherin, RHOA, RET, KIT, and DUSP10 in the VEGFA-VEGFR signalling pathway in placental tissue of obstetric APS patients compared to normal placenta. This suggests that antiphospholipid antibodies induce changes in miRNA expression, which in turn regulate genes in vascular-associated pathways. The study also highlighted the suppression of certain miRNAs, leading to the overexpression of their target genes. Further characterisation of other miRNAs and their target genes is warranted to gain a better understanding of the miRNA-associated mechanisms involved in APS. Functional analysis should be conducted to investigate the associated genes of miR-525-5p, which showed significant downregulation in APS samples. Establishing the relationship between miRNAs and their target genes in placental tissue could contribute to the development of a novel database on miRNA, particularly considering that the targeted genes identified in this study are primarily based on cancer research.

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