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ORIGINAL ARTICLE

MicroRNA profiling in complete and partial hydatidiform moles

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Abstract

Introduction: Hydatidiform mole is one of the gestational trophoblastic disease and comprises complete (CM) and partial moles (PM), which carries a risk of developing persistence disease, invasive mole or choriocarcinoma. MicroRNAs (miRNAs) have been discovered in various tissues, including neoplastic tissues. Its role in the pathogenesis of molar pregnancy or as biomarkers are still largely uncertain. The aim of this study is to identify the differentially expressed miRNAs in CM and PM. *Materials and Methods:* Using next-generation sequencing, the miRNAs profiles of CM (n=3) and PM (n=3) moles, including placenta of non-molar abortus (n=3) as control were determined. The differentially expressed miRNAs between each group were analysed. Subsequently, bioinformatics analysis using miRDB and Targetscan was utilised to predict target genes. *Results:* We found 10 differentially expressed miRNAs in CMs and PMs, compared to NMAs, namely miR-518a-5p, miR-423-3p, miR-503-5p, miR-302a-3p, and miR-1323. The other 5 miRNAs were novel, not listed in the known database. The 3 differentially expressed miRNAs in CMs were predicted to commonly target ZTBT46 and FAM73B mRNAs. *Discussion:* miR-518 was consistently observed to be downregulated in CM versus PM, and CM versus NMA. Further bioinformatic analysis to provide insight into the possible role of these miRNAs in the pathogenesis of HMs, progression of disease and as potential diagnostic biomarkers as well as therapeutic targets for HMs is needed.

Keywords: hydatidiform moles, molar pregnancy, next generation sequencing, microRNA, molecular

INTRODUCTION

Hydatidiform moles (HMs) are characterised by unique genetic make-up as a result of abnormal fertilisation. It composed of abnormal placental villous tissue and represents a premalignant condition as part of the spectrum of gestational trophoblastic disease (GTD). Histologically, HMs demonstrate variable degree of trophoblastic proliferation and villous hydrops. It consists of two main subtypes: complete hydatidiform moles (CMs) and partial hydatidiform moles (PMs). The incidence of HMs varies between 0.5 and 13.0 per 1000 pregnancies. CMs are typically diploid with androgenic-only nuclear genome[1], while over 90% of PHMs are diandric triploid, resulting from fertilization by two sperms with only a minority (<10%) being diploid.

Distinguishing HMs from non-molar abortuses (NMAs) and subclassifying HMs is essential due to varied patient management strategies and clinical prognoses. We reported a diagnostic accuracy of only about 70% for PMs if diagnosis was made without the assistance of ancillary tests such as p57 immunohistochemistry and ploidy analysis.[2] Approximately 15-20% of CMs and 0-5% of PMs progress to post-molar gestational trophoblastic neoplasia (GTN), which may require chemotherapy.[3] CMs exhibit an increased risk of recurrent molar pregnancies, especially heterozygous/dispermic CHMs, which confer a higher risk for gestational trophoblastic disease (GTD) compared to homozygous/ monospermic CMs.[4]

Currently, the combination of p57 immunohistochemistry, ploidy status analysis

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and short tandem repeat genotyping has significantly enhanced diagnostic accuracy of HM.[5] Immunomarkers such as HER2, EGFR and ALK have emerged as having both diagnostic and therapeutic utilities. Immunomarkers serve as biological indicators, could offer quantifiable and cost-effective diagnostic methods, with future potential therapeutic value. Studies have identified new potential useful diagnostic immunomarkers for HMs.[6] Circulating and exosomal microRNAs (miRNAs) have been implicated as potential molecular biomarkers for diseases and cancers. [7]

With the advancement of RNA-seq, it is possible to identify the differentially expressed genes related to placental functions, genetic imprinting, oncogenesis, immune pathways and others in various conditions. MiRNAs are non-coding small RNAs of approximately 21–25 nucleotides in length which function as post-transcriptionally regulators of gene expression, by antisense complimentarity to specific messenger RNAs.[8,9] MiRNAs have tissue-specific expression patterns. MiRNAs are expressed abundantly in the placenta, and change across different gestational age, and are involved in a plethora of placental biological processes.[10]

MiRNAs play a crucial role in governing gene expression, influencing a wide array of biological processes including stem cell maintenance, differentiation and development.[11,12] Their dysregulation has been implicated in various aspects of health and disease, including cancer, placental development, and pregnancy-related complications. In cancer, miRNAs act as tumor suppressors or oncogenes, impacting key processes such as cell growth, cell cycle progression, invasion, metastasis, and angiogenesis.[13] Altered miRNA expression profiles serve as valuable indicators for diagnosis as well as prognostic factors in various types of cancer.[14] In placental development, miRNAs play key roles in trophoblast differentiation, vascularization, syncytialisation, and immune modulation. It may also be involved in pregnancy disorders like preeclampsia.[15] The multifaceted roles of miRNAs in gene regulation underscore the importance of understanding their functions in the molecular pathways behind cancer and other disorders.

Microarray technology is high-throughput but can only detect a limited number of miRNAs because of the nature of probe hybridisation. On the other hand, NGS technology generates short reads (35 bp) but more than 1G bp of sequence data per run and can be used to measure the abundance of small RNA sequences in a sample. As miRNAs are only about 20 bp in length, this technology can enable in-depth characterization of the global repertoire of miRNAs.[16] Hence, NGS is most suited in profiling of miRNAs. The aim of this study is to use NGS in identifying potential miRNA signatures in CM and PM, and evaluating the potential of miRNAs as molecular biomarkers in distinguishing the different subtypes of HMs and their mimics, as well as investigating the role of miRNAs in the pathogenesis of HMs.

MATERIALS AND METHODS

Hydatidiform mole: Formalin fixed paraffin embedded (FFPE) tissue samples

This was a retrospective, cross sectional study of 3 cases of CM, 3 cases of PM and 3 cases NMA as control. The diagnosis of all cases was confirmed by p57 immunohistochemistry and ploidy status analysis using fluorescent in situ hybridisation, as previously described by Wong *et al*.[2] The FFPE tissue blocks were retrieved from the archive of the Department of Diagnostic Laboratory Services, Hospital Canselor Tuanku Muhriz (HCTM). Ethics approval was granted by our institutional human research ethics committee (approval code: JEP-2019-820).

RNA extraction and analysis of the integrity quality

Total RNA was extracted from the FFPE tissue blocks using Qiagen miRNeasy FFPE kit (Qiagen, Germantown, MD, USA; Cat. No: 217504), following the manufacturer's protocol. The RNA concentration and integrity quality were assessed using an Agilent Bioanalyzer 2200 Tapestation system (Agilent Technologies, Palo Alto, CA, USA) and the RNA integrity number (RIN) was used to determine the RNA integrity quality of extraction.

MiRNA library preparation and quantification by NGS sequencing

Nine libraries of RNA-seq were constructed using QIAseq miRNA Library Kit (QIAGEN, Germantown, MD, USA; Cat. No: 331505), according to manufacturer's protocol. Briefly, 3'- and 5'-adapters were ligated to RNA templates, followed by cDNA synthesis and cDNA clean-up. The cDNA was then amplified to prepare the miRNA library. Library concentration was

quantified by qPCR Quant Kit using VAHTS Library Quantification Kit for Illumina NQ-101 (Vazyme Biotech, PRC), and the insert size was checked on an Agilent Bioanalyzer 2200 Tapestation System (Agilent Technologies, Palo Alto, Santa Clara, CA, USA). The cDNA libraries were pooled into a single tube and were sent for next generation sequencing using the Illumina HiSeq platform by the Azenta Life Science.

Analysis of the miRNAs expression and differentially expressed miRNAs between CM, PM and NMA

The original small RNA reads were processed with Trimmomatic (V0.30), Cutadapt (V1.3) and Fast-QC (V0.10.1) to remove adaptor sequences and low-quality sequences. The small RNA reads were mapped with bowtie2 (V2.1.0). The known and novel miRNAs were predicted using the miRDeep2 (V2_0_0_8) and annotated using Blast (V2.2.28+). Differential expression of miRNAs analysis was done using DESeq (V1.6.3), DESeq (V1.18.0) and EdgeR (V3.4.6). Results are expressed as means ± SE and were compared using paired t-test. Results are expressed as means ± SE and were compared using paired t-test. MiRNAs were considered as differentially expressed according to the following criteria: p-values <0.05, false discovery rate (FDR) <0.05, and fold change (Log2FC) >1 or <0.05. For miRNA target gene prediction, miRDB (v6.0) and TargetScan (v8.0) softwares were used to discover the potential miRNA targets. The softwares used for the bioinformatics analysis are listed in Table 1.

RESULTS

Patients' clinicopathological characteristics

There was a total of 9 cases in this study, comprised 3 CMs, 3 PMs and 3 NMAs. The diagnosis of all cases was confirmed by p57 immunohistochemistry and ploidy DNA analysis. Table 2 shows the clinicopathological data, p57 and ploidy analysis status.

Analysis of differentially expressed miRNAs in CM, PM and NMA

Based on principal component analysis (PCA) plot, one of the samples in CM was deemed an outlier and was excluded from the study (Fig. 1). In total, NGS detected 981 miRNAs in CMs, 1,231 miRNAs in PMs, and 1,108 miRNAs in NMAs. Among 3,320 human miRNAs identified, 83 miRNAs were found to be differentially expressed in the 3 groups ($p < 0.05$ and $0.05 > \text{Log2FC} > 1$); 60 of them were downregulated while 23 were upregulated. The volcano plots in Fig. 2 shows 29 miRNAs from CMs, 24 miRNAs from PMs and 7 miRNAs from NMAs were downregulated, while 7 (CMs), 7 (PMs) and 5 (NMAs) miRNAs were significantly upregulated, respectively.

Notably, 10 of the 83 differentially expressed miRNAs showed significant FDR <0.05. An FDR adjusted p-value (or q-value) of 0.05 implies that 5% of significant tests will result in false positive. Table 3 shows the average transcript per million (TPM) count of the 10 significant differentially expressed miRNAs in each sample group, while the dysregulation of miRNA expression and chromosomal location of the miRNAs are shown in Table 4.

TABLE 1: Software used in the bioinformatics analysis of miRNA NGS results

Analysis	Software	Version
Quality control (QC)	Trimmomatic	V0.30
	Cutadapt	V1.3
QC assessment	FastQC	V0.10.1
Mapping	Bowtie2	V2.1.0
microRNA prediction	miRDeep2	V2_0_0_8
Annotation	Blast	V2.2.28+
microRNA target gene prediction	miRDB	6.0
	TargetScan	8.0
Different expression gene analysis	DESeq2	V1.6.3
	DESeq	V1.18.0
	EdgeR	V3.4.6

TABLE 2: Clinicopathological features of patients with complete mole, partial mole and non-molar abortus

	Maternal age	Gestational age	β-hCG level (mIU/mL)	P57 status	Ploidy Status	Final diagnosis
1	37	8	30421.5	negative	Diploid XX	CM
2	40	8	419644.8	negative	Diploid XX	CM
3	28	10	395966.1	negative	Diploid XX	CM
4	27	NA	26917.6	positive	Triploid XXY	PM
5	27	NA	NA	positive	Triploid XXY	PM
6	36	16	38112.9	positive	Triploid XXY	PM
7	36	12	NA	positive	Diploid XX	NMA
8	43	10	67770.5	positive	Diploid XX	NMA
9	38	11	239.6	positive	Diploid XX	NMA

CM – complete mole, PM – partial mole, NMA – non-molar abortus, NA – not available

Intriguingly, five of the 10 significant differentially expressed miRNAs were known miRNAs in the miRNA databases, while the remaining five were novel miRNAs. The 5 known miRNAs that were dysregulated in HMs consists of hsa-miR-518a-5p, hsa-miR-423-3p, hsa-miR-503-5p, hsa-miR-320a-3p and hsa-miR-1323. Of the 5 known miRNAs, 3 miRNAs (hsa-miR-518a-5p, hsa-miR-423-3p, and hsa-miR-503-5p) were downregulated in CMs, while hsa-miR-320a-3p was upregulated in PMs and hsa-miR-1323 was downregulated in NMAs. As for the 5 novel

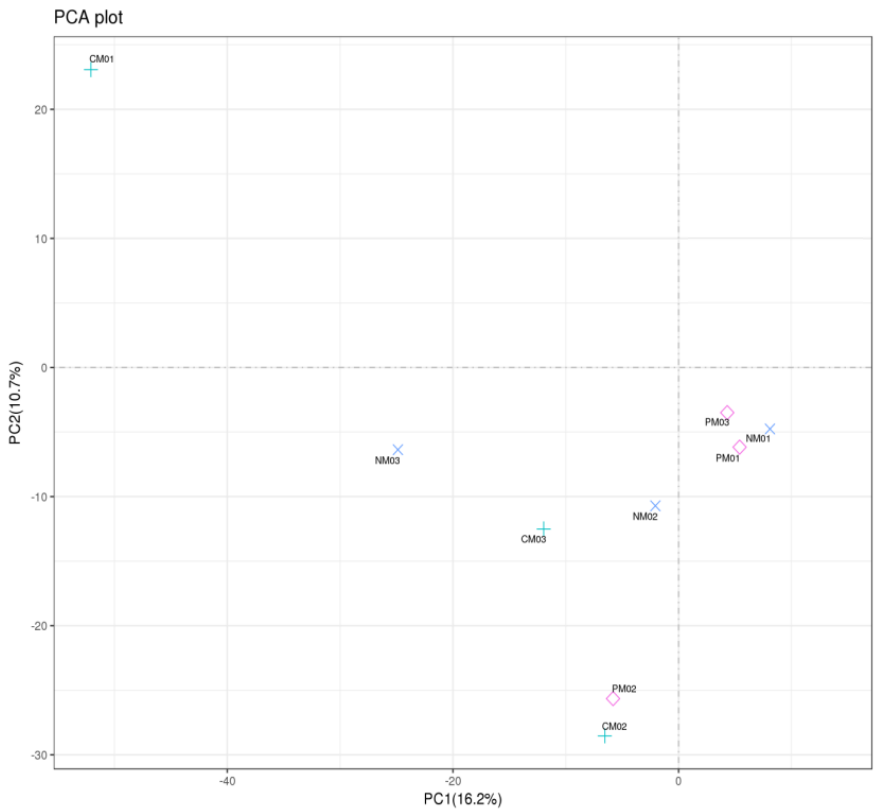
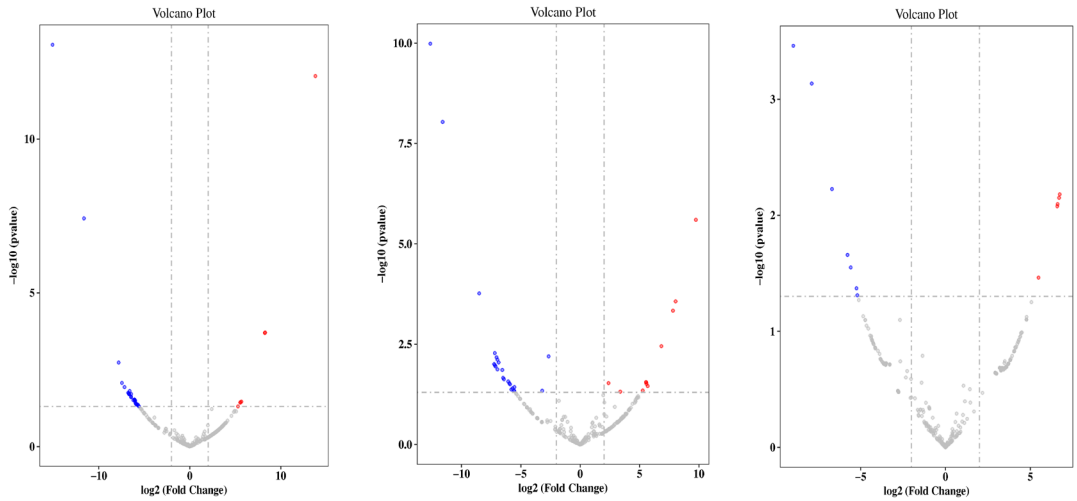


Fig. 1: Principal component analysis plot of microRNAs in hydatidiform mole (complete mole and partial mole) and non-molar abortus tissue samples. One of the complete mole samples (at left upper quadrant) was excluded from the bioinformatic analysis as it was considered an outlier.



(a) CM vs NMA (b) CM vs PM (c) PM vs NMA

Fig. 2: Volcano plots showing a) 36 differentially expressed miRNAs between complete moles (CMs) and non-molar abortuses (NMAs), b) 35 differentially expressed miRNAs between CMs and partial moles (PMs), and c) 12 differentially expressed miRNAs between PMs and NMAs. Each circle dot represents fold change and p-value of different miRNAs. Horizontal dashed line indicates p-value=0.05. Above values from this horizontal line indicates p-value <0.05 which was considered statical significance. Blue dots represent significant downregulated miRNAs while red dots represent upregulated miRNAs in each group.

miRNAs (NovelmiRNA-119, NovelmiRNA-144, NovelmiRNA-318, NovelmiRNA-455 and NovelmiRNA-499), we tried to match them for any similarity in the miRbase, and the results are listed in table 5.

Prediction of mRNA targets using miRDB and Targetscan databases

The target mRNAs of differentially expressed miRNAs were predicted using the TargetScan and miRDB online analysis tools. TargetScan predicted a total of 5,409 target mRNAs for mir-518a-5p, 17 targets for mir-423-3p, 311 targets for mir-503-5p, 1,019 targets for mir-302-3p and 5,731 targets for mir-1323 while miRDB predicted a total of 1,049 target mRNAs for mir-518a-5p, 30 targets for mir-423-3p, 380 targets for mir-503-5p, 1,045 targets for mir-302-3p and 1,145 targets for mir-1323 (Table 6). Venn diagrams were generated to highlight the common mRNA targets predicted by miRDB and TargetScan analysis softwares in downregulated miRNAs in CM (Fig 3). Both miRDB and TargetScan predicted one common mRNA target each between mir-518a-5p, mir-423-3p and mir-503-5p, namely ZBTB46 and FAM73B, respectively. A complete list of target mRNA of all 5 differentially expressed miRNAs is listed in Supplementary Table 1.

DISCUSSION

Gestational trophoblastic disease (GTD) encompasses CM and PM, and has risk to progress into gestational trophoblastic neoplasia, of which the most worrisome is choriocarcinoma. Currently, there are a number of studies that investigated the use of miRNAs as biomarker of progression of GTD. The current study identified 10 differentially expressed miRNAs between GTD and NMA using NGS. Among these, five were known miRNAs and the remaining five were novel discoveries. Within the known miRNAs, three demonstrated downregulation in CMs (miR-518a-5p, miR-423-3p, and miR-503-5p), one was downregulated in NMAs (miR-1323), and the other exhibited upregulation in PMs (miR-320a-3p).

Among the known miRNAs identified in our study, miR-518a-5p and miR-1323 were located on chromosome 19q13.41, which are commonly referred to as the chromosome 19 microRNA cluster (C19MC). The C19MC is one of the largest miRNA gene clusters in human and spans a 100 kb long region. It encompasses 46 conserved miRNAs that are primate-specific and conserved in humans.[17] Notably, C19MC exhibits imprinting in the placenta, with expression originating from the paternally inherited chromosome.[18] Researchers have explored the expression of C19MC to identify miRNAs

TABLE 3: Lists of significantly differentially expressed miRNAs in complete moles, partial moles and non-molar abortuses

a) CHM vs PHM

miRNA	Average TPM		Log2FoldChange	p-value	Adjusted p-value	Regulation
	CHM	PHM				
hsa-miR-518a-5p	0	2,527.60	8.036467193	0.000	0.006	Upregulated in PM
hsa-miR-423-3p	0	2,194.03	7.813529291	0.000	0.008	Upregulated in PM
hsa-miR-503-5p	0	2,469.48	5.685082394	0.035	0.1533	Upregulated in PM
hsa-miR-320a-3p	0	10,965.93	9.74207202	0.000	0.0001	Upregulated in PM
NovelmiRNA-318	18134.58	0	-11.58493329	0.000	0.0000	Downregulated in PM
NovelmiRNA-499	40892.76	0	-12.62314678	0.000	0.0000	Downregulated in PM
NovelmiRNA-119	1673.67	0	-8.505836419	0.000	0.0047	Downregulated in PM

b) PHM vs NMA

miRNA	Average TPM		Log2FoldChange	p-value	Adjusted p-value	Regulation
	PHM	NMA				
NovelmiRNA-455	278,709.49	0	-8.963604242	0.000	0.02765	Downregulated in NMA
hsa-miR-1323	1,480.14	0	-7.879944861	0.001	0.02925	Downregulated in NMA
NovelmiRNA-119	0	300.90	6.691569557	0.007	0.09558	Upregulated in NMA

c) CHM vs NMA

miRNA	Average TPM		Log2FoldChange	p-value	Adjusted p-value	Regulation
	CHM	NMA				
hsa-miR-518a-5p	0	2,396.30	8.278943111	0.000	0.0021	Upregulated in NMA
hsa-miR-423-3p	0	1,200.72	5.672090568	0.035	NA	Upregulated in NMA
hsa-miR-503-5p	0	2,633.45	8.235386054	0.000	0.0021	Upregulated in NMA
hsa-miR-1323	1,586.75	0	-6.20609057	0.031	NA	Downregulated in NMA
NovelmiRNA-318	18,134.58	0	-11.62615689	0.000	0.0000	Downregulated in NMA
NovelmiRNA-455	356,688.60	0	-15.07791071	0.000	0.0000	Downregulated in NMA
NovelmiRNA-144	0	244,318.91	13.7944619	0.000	0.0000	Upregulated in NMA

TABLE 4: Dysregulation of microRNAs' expression and chromosomal location of miRNA

MicroRNAs	Types of dyregulations	Chromosomal location
hsa-miR-518a-5p	Downregulated in CM	Chromosome 19
hsa-miR-423-3p	Downregulated in CM	Chromosome 17
hsa-miR-503-5p	Downregulated in CM	Chromosome X
hsa-miR-320a-3p	Upregulated in PM	Chromosome 8
hsa-miR-1323	Downregulated in NMA	Chromosome 19
NovelmiRNA-318	Upregulated in CM	Chromosome 10
NovelmiRNA-499	Downregulated in PM	Chromosome 2
NovelmiRNA-119	Downregulated in PM	Chromosome 9
NovelmiRNA-455	Downregulated in NMA	Chromosome 19
NovelmiRNA-144	Upregulated in NMA	Chromosome 20

TABLE 5: A comparison of similarity of novel miRNA with listed miRNA in miRbase database

Novel miRNA	Number of hits	Similar identity	Identity similarity (%)	Query coverage (%)
NovelmiRNA-119	4	URS000075A37B_9606 Homo sapiens (human) hsa-miR-5691	91.67	54.55
		URS00005DA9AC_9606 Homo sapiens (human) hsa-miR-4714-5p	80.00	68.18
		URS000075C704_9606 Homo sapiens (human) hsa-miR-6841-5p	76.92	59.09
		URS000075B11C_9606 Homo sapiens (human) hsa-miR-7976	78.57	63.64
NovelmiRNA-144	2	URS000053E475_9606 Homo sapiens (human) hsa-miR-3691-3p	81.25	76.19
		URS000075C639_9606 Homo sapiens (human) hsa-miR-632	100.00	57.14
NovelmiRNA-318	2	URS000075CEFF_9606 Homo sapiens (human) microRNA hsa-mir-650 precursor	86.67	62.50
		URS000075DEB1_9606 Homo sapiens (human) microRNA hsa-mir-4656 precursor	83.33	75.00
NovelmiRNA-455	1	URS00000EE7F0_9606 Homo sapiens (human) hsa-miR-544b	85.71	60.87
NovelmiRNA-499	2	URS000075CFA3_9606 Homo sapiens (human) hsa-miR-5197-3p	84.62	56.52
		URS0000550C66_9606 Homo sapiens (human) hsa-miR-452-5p	83.33	52.17

TABLE 6: Predicted targets of mRNAs of differentially expressed miRNAs using the TargetScan and miRDB online analysis tools

miRNA	Number of predicted target mRNAs	
	TargetScan	miRDB
mir-518a-5p	5,409	1,049
mir-423-3p	17	30
mir-503-5p	311	380
mir-302-3p	1,019	1,045
mir-1323	5,731	1,145

with diagnostic potential, revealing their high expression in placenta tissue, trophoblastic cells, and maternal plasma.[19] However, the regulation of expression of C19MC miRNAs still is poorly understood.

The expression of C19MC miRNAs is reported to be high in trophoblast cells isolated from first trimester placentas and trophoblast cells isolated from normal term placentas.[19] However, miR-518a-5p was downregulated in CM in the current study. Similarly, Na *et al.*[20] reported that miR-517a, miR-517b, miR-518b and miR-519a were down-regulated in CMs compared to normal villous tissues. These miRNAs are closely located close to each other in the genome on chromosome 19. Our sequencing results reaffirm this finding.

MiR-1323 is one of the miRNAs in the C19MC; it was reported to be highly expressed in placenta tissues and trophoblast cells isolated from first trimester placentas.[21,22] We found that MiR-1323 was downregulated in NMAs in the first trimester as compared to CMs and PMs. Gillet *et al.*[23] reported that the expression of miR-1323 was significantly upregulated in patients with gestational diabetes mellitus. Another study reported downregulation of miR-1323 in placentas of pregnancies with intrauterine growth restriction compared to placenta of uncomplicated pregnancies. Maternal serum miR-1323 was also found to be downregulated in pregnancy associated with foetal growth restriction.[16]

Along with miR-424(322), this miR-503-5p constitutes a mammal-specific segment of

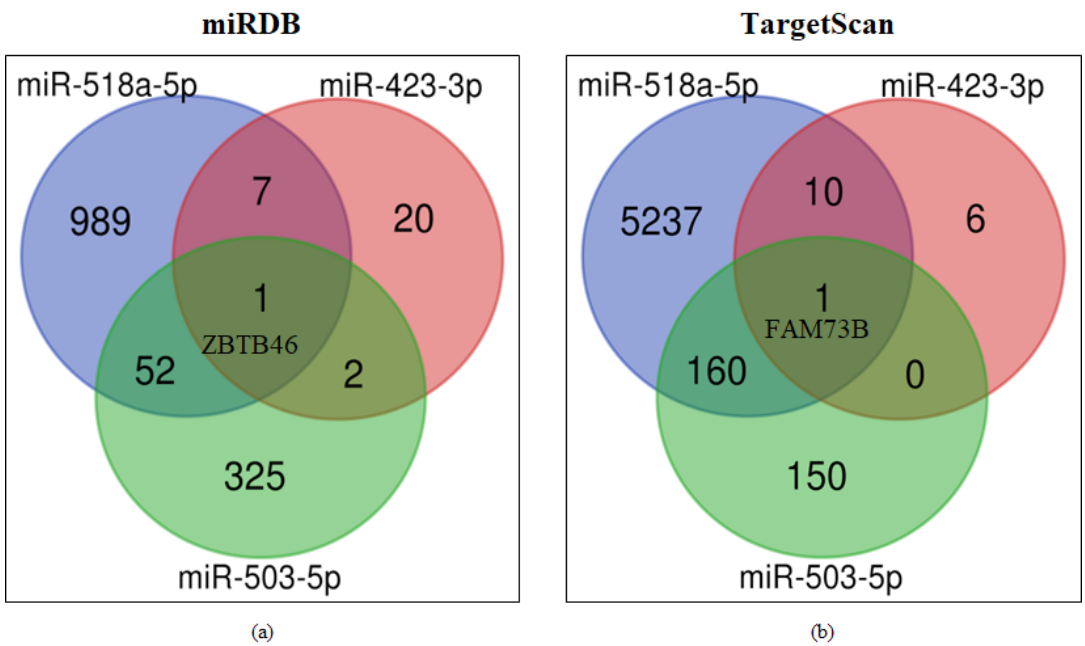


Fig. 3: Venn diagrams show one common target mRNAs from the 3 differentially expressed miRNAs that were downregulated in complete moles, predicted by a) miRDB database (ZBTB46) and b) TargetScan database (FAM73B)

the miR-15/107 microRNA family.[24] It plays a pivotal role in placental development[25] and contributes to fundamental cellular processes, including cell cycle regulation, epithelial-to-mesenchymal transition, hypoxia response, and tissue differentiation and remodelling.[25] Meanwhile, miR-423-3p and miR-320-3p have been implicated in placenta-related complications. miR-423-3p has possible role in early-onset preeclampsia.[26] Timofeeva *et al*.[27] suggested that combining the assessment of the secretory form of clusterin with the expression levels of several specific miRNAs, including miR-320a-3p, in the plasma of pregnant women could serve as potential predictors for different forms of placenta accreta spectrum.

Zinc finger and BTB domain containing 46 (ZBTB46), located in chromosome 20, is a transcription factor identified in dendritic cells. There are >1300 potential gene targets of ZBTB46, that could affect many physiological processes including cell cycle. ZBTB46 expression was noted in endothelial cells of the splenic vasculature. Wang *et al*.[28] described ZBTB46 inhibits endothelial cells proliferation and may contribute to many disease processes such as atherosclerosis, pulmonary hypertension, postsurgical neointimal hyperplasia, in-stent restenosis, vein bypass graft failure, and transplant vasculopathy. They also found that cells overexpressing ZBTB46 showed significantly less frequency of Ki67-positive cells. It can inhibit the proliferation of endothelial cells in vitro, and exert its anti-proliferative effects through regulation of genes involved in the cell cycle. We found that the miRNAs that target ZBTB46 were downregulated in CMs. Therefore, in CMs theoretically, the predicted target ZBTB46 may be overexpressed, resulting in inhibition of endothelial cells proliferation. As CMs lack or have few blood vessels in the hydropic villi, this could be regulated by ZBTB46.

The other predicted mRNA target was the family with sequence similarity 73, member B (FAM73B), also known as mitoguardin 2 (MIGA2). It is a mitochondrial outer membrane protein. A study reported that FAM73B deficient mice were resistant to tumour growth. Hence, it negatively regulates anti-tumour innate immunity. Moreover, FAM73B deletion in mice with melanoma suppressed tumour growth and improved survival rate of the mice.[29] Unregulated overexpression of FAM73B in CMs may result in loss of antitumour immunity.

CONCLUSION

Our study found 10 differentially expressed miRNAs between CM, PM and NMA. Notably, miR-518 was consistently observed to be downregulated in CM versus PM, and CM versus NMA. In addition, miR-518 was also detected in other studies, in particularly CM with progression to gestational trophoblastic neoplasia. This study is limited by the small number of cases subjected to sequencing. Further bioinformatic analysis to provide insight into the possible role of these miRNAs in the pathogenesis of HMs, progression of disease and as potential diagnostic biomarkers as well as therapeutic targets for HMs is needed.

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