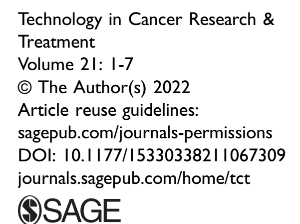
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MicroRNA Expression Profiling in Hydatidiform Mole for the Prediction of Postmolar GTN
MicroRNA Profile in Postmolar GTN

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Abstract

Objectives: The primary aim of the study was to identify miRNAs that were differentially expressed between complete hydatidiform moles (CHMs) that turned out to be gestational trophoblastic neoplasia (GTN) [GTN moles] and CHMs that regressed spontaneously after evacuation [remission moles]. The secondary aim was to study the profiles of miRNA expressions in CHMs. **Methods:** A case-control study was conducted on GTN moles and remission moles. We quantitatively assessed the expression of 800 human miRNAs from molar tissues using Nanostring nCounter. **Results:** From a pilot study, 21 miRNAs were significantly downregulated in GTN moles compared to the remission moles. Five of them (miR-566, miR-608, miR-1226-3p, miR-548ar-3p and miR-514a-3p) were downregulated for >4 folds. MiR-608 was selected as a candidate for further analysis on 18 CHMs (9 remission moles and 9 GTN moles) due to its striking association with malignant formation. MiR-608 expression was slightly lower in GTN moles compared to the remission moles, that is, 2.22 folds change [$p = 0.063$]. **Conclusion:** We identified 21 miRNAs that were differentially expressed between GTN moles and remission moles suggesting that miRNA profiles can distinguish between the two groups. Although not reaching statistically significant, miR-608 expression was slightly lower in GTN moles compared to remission moles.

Keywords

postmolar GTN, microRNA, nanostring

Introduction

Gestational trophoblastic disease (GTD) is a pregnancy-related disorder originating from abnormal proliferation of the placental trophoblasts. It can be classified as benign disorders or hydatidiform moles and malignant disorders or gestational trophoblastic neoplasia (GTN) of which the latter includes the invasive mole, choriocarcinoma, placental-site trophoblastic tumor and epithelioid trophoblastic tumor.[1,2] Generally, hydatidiform moles would regress after evacuation, but about 15% of complete hydatidiform moles (CHMs) and 0.5 to 1% of partial hydatidiform moles (PHMs) could progress into postmolar GTN.[2] The risk of developing postmolar GTN varies among different regions in the world. In Thailand, there is a higher rate of developing postmolar GTN which was approximately 26%.[3]

Prophylactic chemotherapy may reduce the risk of progression to postmolar GTN. However, this practice is not generally recommended because it may increase drug resistance, delays treatment, and most importantly, it may expose women to unnecessary toxic side effects.[4] In order to detect postmolar GTN, several hCG regression models have been used.[5] To use these models, careful monitoring with frequent follow-up

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visits needs to be done. Therefore, identifying patients who would have postmolar GTN at the time of evacuation would be of great value because intensive monitoring can be planned in selected high-risk patients.

Another biomarker, endocrine-gland derived vascular endothelial growth factor (EG-VEGF), which is an angiogenic factor that controls trophoblastic proliferation, was found to be regulated by hCG and was increased in choriocarcinoma and CHMs which led to the idea that it could be a target for the treatment of choriocarcinoma.[6,7] However, it has not yet been identified as a predictive marker for postmolar GTN.

MicroRNAs (miRNAs) are small non-coding RNAs consisting of 18 to 24 nucleotides. These molecules have been shown to regulate translation and stability of mRNAs.[8,9] Dysregulations of miRNAs have been detected in several human cancers such as colon cancer, nasopharyngeal carcinoma, pancreatic cancer, breast cancer and esophageal cancer.[10–14] Differentially expressed miRNAs found in cancers have led to the classification of miRNAs as oncogenic miRNAs and tumor suppressor miRNAs.[9] Moreover, some of these dysregulations have been found during the precancerous period, for example, colon adenoma.[14]

There are limited data of the profile of miRNAs in GTD. Some miRNAs including miR-517a, miR-517b, miR-518b and miR-519a were found to be under expressed in CHMs compared to normal villous tissues.[15] Other unique miR-520 clusters were upregulated in CHMs compared to normal villous tissues, and the levels of these miRNAs also changed, following the pattern of β hCG levels after evacuation of CHMs.[16,17] Another miRNA, miR-199b, along with few other miRNAs were found under expressed in choriocarcinoma compared to non-cancer trophoblasts and hydatidiform moles.[18] There are limited data on miRNA expression in predicting postmolar GTN. A distinct microRNA profile of CHMs progressing to GTN was reported in one study suggesting miR-181 family members to be potential prognostic biomarkers for predicting GTN.[19]

Therefore, we assessed the miRNAs that were differentially expressed between CHMs that turned out to be GTN (GTN moles) and CHMs that regressed spontaneously after evacuation (remission moles), and we also studied the profiles of miRNA expression in CHMs.

Materials and Methods

This study was a case-control study conducted at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Ethical approval was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No.577/59). The medical records of all patients with pathologically confirmed CHMs diagnosed between January 2007 and December 2016 were reviewed. Cases of CHMs with formalin-fixed paraffin embedded (FFPE) or fresh frozen specimens kept at the division of Gynecologic Pathology at King Chulalongkorn Memorial Hospital who completed clinical follow-up periods of at least one year were included in the study. The cases and controls were matched by age and year at diagnosis. All specimens were pathologically reassessed by two pathologists. Cases with low quality specimens or inadequate tumor tissues for RNA extraction were excluded from the study. The diagnosis of postmolar GTN was based on the International Federation of Gynecology and Obstetrics (FIGO) 2000 criteria.

RNA Extraction

Total RNA was extracted from formalin fixed, paraffin embedded (FFPE) or fresh frozen tissue using RNeasy FFPE kit according to the manufacturer's instruction (Qiagen, Germantown, MD) and performed at Chula GenePRO Center, Faculty of Medicine, Chulalongkorn University. The lesions of interest (villous tissues) were reviewed and selected by the pathologist. Three to five ribbons of 10-μm FFPE tissue section were used for total RNA extraction. Then, RNA concentration and quality were determined by using nanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The RNA was stored at -80°C until miRNA expressions were analyzed.

miRNA Expression Analysis

Analysis of miRNA expression was performed using nCounter Analysis System and Human v3 miRNA Assay kit according to the manufacturer's instruction (nanoString, Seattle, WA). One hundred nanograms of each total RNA samples were mixed with the probes and incubated at 65°C for 16 h. Then probe-hybridized samples were processed on the nanoString prep station. The processed cartridges were then transferred to the nanoString digital analyzer and scanned on HIGH mode for 280 fields of view per samples. The data quality control was performed using default parameters of miRNA analysis. The raw data counts were subtracted with median of the counts of negative control probes. Then the data were normalized using the geometric mean of *GAPDH* expression. The data QC, background subtraction and normalization were performed using nSolver Analysis Software 4.0 (nanoString Technologies Inc., Seattle, WA). MiRNAs differential expression was visualized using volcano plot performed on GraphPad Prism version 9.2.0 (GraphPad Software, CA, USA). Heat-map was used for data visualization and two-way hierarchical clustering was performed on both significantly altered miRNAs and cases using nSolver Analysis Software 4.0 (nanoString Technologies Inc., Seattle, WA).

Sample Size Calculation and Statistical Analysis

A pilot study of 10 FFPE specimens of CHMs (5 remission moles and 5 GTN moles) was done to discover the differentially expressed miRNA candidates between remission moles and GTN moles. Sample size calculation was based upon the mean difference and standard deviation of the count of

Table 1. Differentially expressed miRNAs between GTN moles group and the remission moles group.

miRNA	GTN (N = 5)	Remission (N = 5)	Fold change (Downregulated)	p value
hsa-miR-1185-2-3p	1.89 (1.16, 2.40)	3.23 (2.51, 6.67)	1.71 (1.13-4.59)	0.028
hsa-miR-1226-3p	1.32 (1.00, 3.38)	6.07 (3.67, 7.38)	4.60 (1.43-7.26)	0.028
hsa-miR-1270	1.09 (1.00, 1.69)	4.33 (2.19, 7.45)	3.97 (1.33-7.53)	0.016
hsa-miR-136-5p	1.00 (1.00, 1.69)	2.23 (1.76, 2.90)	2.23 (1.19-2.83)	0.026
hsa-miR-187-3p	2.06 (1.00, 3.89)	7.59 (3.98, 9.92)	3.68 (1.28-8.14)	0.028
hsa-miR-195-5p	1.64 (1.16, 2.29)	3.23 (2.14, 4.24)	1.97 (1.02-3.33)	0.047
hsa-miR-196a-5p	5.95 (3.79, 9.95)	12.90 (11.40, 18.44)	2.17 (1.26-4.33)	0.028
hsa-miR-200b-3p	1.32 (1.00, 1.98)	2.75 (1.76, 4.87)	2.08 (-1.01-4.11)	0.047
hsa-miR-23b-3p	5.03 (3.38, 9.68)	10.63 (8.57, 16.93)	2.11 (-1.05-5.3)	0.047
hsa-miR-30d-5p	6.56 (3.35, 9.48)	19.35 (14.43, 26.36)	2.95 (1.56-7.74)	0.009
hsa-miR-30e-5p	8.76 (4.49, 10.69)	19.35 (12.02, 23.83)	2.21 (1.19-5.16)	0.016
hsa-miR-324-3p	4.63 (1.45, 5.43)	8.06 (5.17, 9.86)	1.74 (-1.17-6.16)	0.047
hsa-miR-495-3p	2.74 (1.97, 4.21)	6.65 (4.75, 12.73)	2.43 (1.16-5.6)	0.028
hsa-miR-514a-3p	2.74 (1.49, 3.94)	11.16 (7.39, 12.52)	4.07 (1.82-8.49)	0.009
hsa-miR-548ar-3p	1.32 (1.00, 5.38)	5.70 (3.65, 11.23)	4.32 (1.02-9.96)	0.047
hsa-miR-566	1.26 (1.05, 1.69)	6.69 (1.75, 9.92)	5.31 (1.07-11.31)	0.028
hsa-miR-574-5p	13.22 (10.39, 21.86)	29.01 (24.12, 86.31)	2.19 (1.16-6.76)	0.028
hsa-miR-608	1.32 (1.00, 2.29)	6.07 (4.38, 10.58)	4.60 (2.15-8.83)	0.009
hsa-miR-640	2.06 (1.16, 2.98)	6.69 (5.13, 8.93)	3.25 (1.90-6.65)	0.009
hsa-miR-98-5p	1.32 (1.00, 2.92)	4.33 (2.38, 5.93)	3.28 (1.00-5.49)	0.047
hsa-miR-99a-5p	1.26 (1.05, 1.69)	1.90 (1.55, 6.69)	1.51 (-1.42-5.91)	0.047

Data presented as median (IQR) and median fold change (95%CI).

miRNA candidate discovered from the pilot study. After the pilot study, miR-608 was chosen as a miRNA candidate. A total of 18 samples (9 remission moles and 9 GTN moles) were required for analysis of miR-608 to get 80% power with an alpha error of 0.05.

Statistical analysis was performed using SPSS version 22 (SPSS Inc, Chicago, IL, USA). Baseline clinical data were presented as mean, median and percentage. Level of expression of each miRNA was presented as median count. Fold change of each miRNA was calculated using the median normalized counts of the probe between remission moles and GTN moles. The differentially expressed miRNAs between GTN moles and remission moles were analyzed using Mann-Whitney U test. A p value of <0.05 was considered as statistically significant.

Results

Pilot Study: Quantitative Assessment of miRNA Profile

In the pilot study, 5 remission moles and 5 GTN moles were analyzed. Among 800 human miRNAs evaluated, 21 miRNAs were found differentially expressed in the two groups ($p < 0.05$), all of which were downregulated. Table 1 shows the median count of each of the differentially expressed 21 miRNAs found in the pilot study. The volcano plot of 800 miRNAs indicated that GTN moles were associated with downregulation of all of the significantly alter miRNAs (Figure 1). Clustering analysis of these 21 miRNAs demonstrated a tree with obvious distinction between remission moles and GTN moles (Figure 2). The largest differences (more than 4 folds downregulations) were seen in 5 miRNAs: miR-566, miR-608, miR-1226-3p, miR-548ar-3p and miR-514a-3p.

Among these 5 miRNAs, miR-566 demonstrated the largest difference with a median count of 1.26 (1.05, 1.69) in GTN moles versus 6.69 (1.75, 9.92) in remission moles ($p = 0.028$). MiR-608 had a median count of 1.32 (1.00, 2.29) in GTN moles versus 6.07 (4.38, 10.58) in remission moles ($p = 0.009$), and miR-1226-3p had a median count of 1.32 (1.00,3.38) in GTN moles versus 6.07 (3.67, 7.38) in remission moles ($p = 0.028$).

After reviewing the functions of these 5 miRNAs, we selected miR-608 as our miRNA candidate for differentiating between remission moles and GTN moles because previous studies have shown it to be strongly associated with malignant formation.[20–23] Further RNA extraction and miRNA analysis were made on 4 more remission moles and 4 more GTN moles in order to get enough sample size with 80% power to validate miR-608 as a differentially expressed miRNA between remission moles and GTN moles. Validating study was also performed on another 4 miRNAs with the largest differences between GTN moles and remission moles in the pilot study.

Patients' Characteristics

Eighteen samples (9 remission moles and 9 GTN moles) were analyzed. Baseline clinical data are shown in Table 2. The age and gestational age of the patients in both groups were similar. Median age of the patients was 30 years (26.5, 40.0) in the remission moles group and 32 years (21.5, 41) in the GTN moles group. Most of the cases were diagnosed with

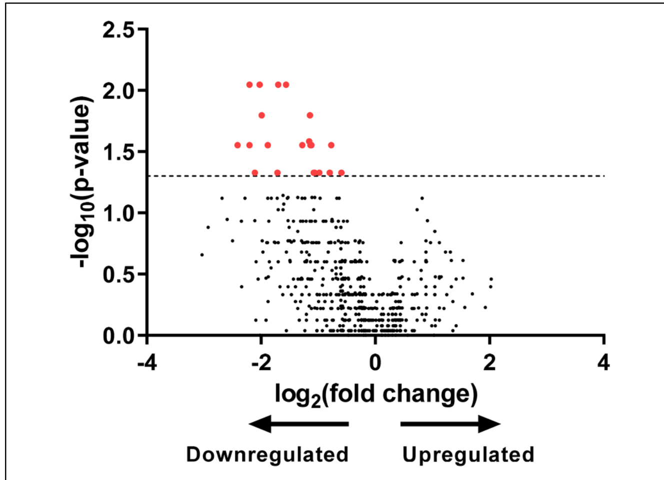

Figure 1. Volcano plot showing nanoString 800 human miRNAs differential expression between GTN moles and Remission moles. Each circle dot represents fold change and p-value of different miRNAs. Horizontal dashed line indicates p-value = 0.05. Above values from this horizontal line indicates p-value < 0.05 which is considered statical significance. Red circle dots represent 21 significant downregulated miRNAs (listed in Table 1) in GTN moles.

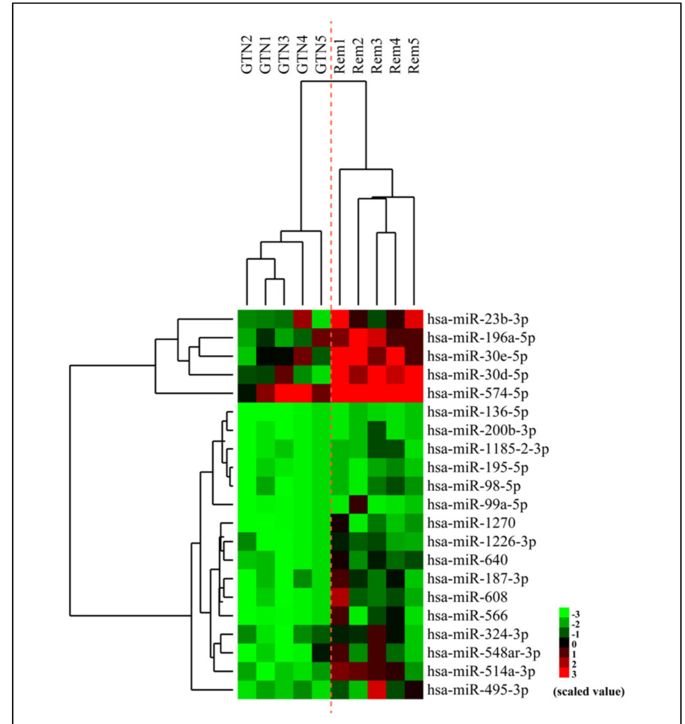

Figure 2. Clustering analysis showing the levels of miRNAs in GTN moles group and the remission moles group. The heat-map shows high (red) to low (green) expression levels of miRNAs in each samples.

molar pregnancy in their first trimester. Pretreatment β hCG was higher in GTN moles group [525020 mIU/ml (160511, 1000000) versus 125693 mIU/ml (74687, 189566)]. The patients in both groups were treated mostly with suction curettage (77.8%). In the GTN moles group, most of the patients were diagnosed with GTN with the median duration of 45 days (11.5, 56) after treatment of CHMs. The median β hCG level upon diagnosis of GTN was 6367 mIU/ml (1850, 53063). The patients in the remission moles group had a median duration to normal β hCG of 77 days (60.8, 87.5).

Validating miR-608 Candidate

The miR-608 count of all 18 samples were collected. Background subtraction, spike-in-control normalization and reference genes normalization were reperformed using nSlover™ Analysis software 3.0 (nanoString Technologies, Seattle, WA). MiR-608 expression was slightly lower in the GTN moles group compared to the remission moles group by 2.22 folds change and the miRNA count was 2.25 (1.00, 4.17) versus 5.00 (2.90, 5.45), respectively[p = 0.063].

Validating study of all 5 miRNAs with the largest differences between GTN moles and remission moles detecting from the pilot study was shown in Table 3. Among these 5 miRNAs, the median expressions were all downregulated in GTN moles group similar to the pilot study. However, the differences did not reach statistical significance.

Discussion

The molecular basis in GTD is not clearly understood. Evaluation of the molecular pathway driving the malignant risk in CHM is challenging. In the current study, we aimed to evaluate miRNA as a new biomarker for the prediction of post-molar GTN.

This study clearly demonstrated the differences in the profile of miRNA expression between GTN moles and remission moles. Our preliminary analysis showed that 21 miRNAs were significantly downregulated in GTN moles of which, 5 miRNAs (miR-566, miR-608, miR-1226-3p, miR-548ar-3p and miR-514a-3p) demonstrated > 4 folds downregulation. Therefore, we reviewed literatures on the functions of these 5 miRNAs and their associations with malignancy.

MiR-608 is a short non-coding RNA on chromosome 10. In vitro, overexpression of miR-608 was found to target migration inhibitory factor (MIF), suppress proliferation and arrest cell cycle in G0/G1 phase. In vivo, miR-608 was found to be downregulated in many malignant tumors including hepatocellular carcinoma, colon cancer, lung cancer, bladder cancer, glioblastoma and chordoma.[20–23] Our pilot study demonstrated 4.6 folds downregulation of miR-608 in GTN moles group compared to the remission moles group (p = 0.009).

Next, miR-566 and miR-1226-3p are short non-coding RNAs on chromosome 3. The evidence of miR-566 in malignancy is still conflicting. Xiao B et al. reported that downregulation of miR-566 modulated VEGF by upregulating Von-Hippel-Lindau (VHL) gene that inhibited VEGF function; therefore, inhibition of miR-566 inhibited the invasion and migration of glioblastoma.[24] Whereas, in another study by Drusco A et al. demonstrated that downregulation of miR-566

Table 2. Demographic data and baseline clinical characteristics.

	GTN (N = 9)	Remission (N = 9)
Age (years)	32 (21.5, 41)	30 (26.5, 40.0)
GA (weeks)	11.5 (9.25, 13.5)	12 (10, 17)
Pretreatment β hCG (mIU/ml)	525020 (160511, 1000000)	125693 (74687, 189566)
Treatment	7 (77.8%)	7 (77.8%)
• Suction curettage	2 (22.2%)	2 (22.2%)
• Hysterectomy		
Duration to normal β hCG (days)		77 (60.75, 87.5)
Duration to GTN (days)	45 (11.5, 56)	
hCG at D × GTN (mIU/ml)	6367 (1850, 53063)	
Stage	5 (55.6%)	
• Stage I	0 (0%)	
• Stage II	3 (33.3%)	
• Stage III	0 (0%)	
• Stage IV	1 (11.1%)	
• Missing		
7 (77.8%)		
1 (11.1%)		
1 (11.1%)		
Chemotherapy regimen	4 (44.4%)	
• Methotrexate	2 (22.2%)	
• Actinomycin D	1 (11.1%)	
• EMACO	1 (11.1%)	
• None (Surgical treatment – hysterectomy)	1 (11.1%)	
• Missing		

Data presented as median (IQR).

facilitated lymph node invasion in colon cancer.[11] In our study, miR-566 was found to be 5.31 folds downregulated in GTN moles group compared to the remission moles group ($p = 0.028$). For miR-1226-3p, previous data showed that it is downregulated in breast cancer and colorectal cancer.[25,26] Our study also demonstrated 4.6 folds downregulation of miR-1226-3p in GTN moles group ($p = 0.028$).

MiR-514a-3p, a microRNAs located on X chromosome, was reported in several studies to be downregulated in renal cell carcinoma and melanoma.[27–29] The possible mechanism is that it negatively regulated nuclear factor 1 (NF1) and cyclin-dependent kinase 2 (CDK2), therefore it served as a tumor suppressor role. To date, there are limited data on the remaining miR-548ar-3p. One study showed that overexpression of miR-548ar-3p decreases nuclear paraspeckle assembly transcript 1 (NEAT1) expression and promote apoptosis in breast cancer cell, therefore downregulation of this miRNA could lead to tumor proliferation.[30]

Due to the striking association with malignancy, miR-608 was selected as a candidate for further analysis. MiR-608 is transcribed from Chromosome 10q24.31 locus. This miRNA was previously found to be downregulated in hepatocellular carcinoma (HCC) tissues. Reduced miR-608 expression was associated with tumor size, clinical stage, and tumor differentiation. Also low expression of miR-608 was associated with shorter survival in HCC, suggesting its role in carcinogenesis and tumor development.[21] MiR-608 also suppressed the proliferation and tumorigenesis of bladder cancer cells in vitro and in vivo, supporting its role as a tumor suppressor.[20] Our result was consistent with the previous studies that miR-608 even though the result was not statistically significant but there was a trend toward downregulation of miR-608 in GTN moles compared to remission moles suggesting its role in carcinogenesis of CHM.

The study of miRNA profile in CHM is sparse. One study has evaluated the dysregulated miRNAs in GTN by using microarray and identified hundreds of upregulated and downregulated miRNAs in GTN compared with CHM.[31] Another recent study demonstrates a distinct set of miRNAs associated with GTN. In their study, miR-181 family members which target BCL2 were the most significantly altered miRNAs associated with progression to GTN.[19] However, the miRNAs profile in these two studies are different and different from those seen in our study. We think that this could be explained by the heterogeneity of CHM both clinically and pathologically.

The strength of our study is the use of Nanostring nCounter technology to analyze for the miRNA expression. To detect miRNAs in the FFPE specimens, this technology has its advantages over other methods. We can digitally count miRNAs in FFPE specimens without the need for RNA amplification. Over 800 miRNAs can be assessed in a single reaction that is suitable for cases of GTN which no known miRNA candidate was made before this study. Also, this technology has been proven to accurately assess the expression of miRNAs in both fresh frozen tissues and FFPE specimens.[32,33]

Limitations of our study are small sample size and clinical heterogeneity of the cases in both groups. Another limitation is that the Nanostring nCounter technology covered about 800 miRNAs assessment which was about 1/3 of known

Table 3. Validated result of 5 miRNAs with the largest differences between GTN moles group and the remission moles group.

miRNA	GTN (N = 9)	Remission (N = 9)	Fold change (Downregulated)	p value
hsa-miR-566	1.50 (1.07, 7.74)	5.13 (1.09, 8.33)	3.42 (-1.92-3.94)	0.690
hsa-miR-608	2.25 (1.00, 4.17)	5.00 (2.90, 5.45)	2.22 (-1.10-4.17)	0.063
hsa-miR-1226-3p	2.28 (1.00, 2.99)	3.35 (1.09, 5.85)	1.47 (-1.38-3.06)	0.196
hsa-miR-548ar-3p	3.21 (1.00, 11.90)	3.51 (1.80, 10.81)	1.09 (-2.98-3.80)	0.755
hsa-miR-514a-3p	2.70 (1.61, 5.77)	7.86 (3.81, 11.47)	2.91 (-1.18-6.12)	0.057

Data presented as median (IQR) and median fold change (95%CI).

human miRNAs, so we may have missed some of the distinct miRNAs in predicting GTN.[34] However, due to the rarity of the diseases, limited studies have been performed in this field. Therefore, we primarily aimed to do this study as preliminary research to gather information on the profiles of miRNA in the GTN moles group and the remission moles group which can provide data for further replicative studies in this field.

Conclusion

This study demonstrated the different miRNA profiles between GTN moles and remission moles. Twenty-one miRNAs were differentially expressed in the two groups. MiR-608 expression, which was selected as a candidate for this study, was slightly lower in the GTN moles group compared to the remission moles group. However, the result did not reach statistical significance. Aside from miR-608, there are several other miRNAs that could be potential biomarkers for predicting malignant risk in CHMs. Further studies should be done by using this preliminary data to investigate those miRNAs as diagnostic biomarkers for predicting GTN.

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Author Contributions

W.A., P.T., V.P. and R.L. designed the research. W.A. and R.L. wrote the main manuscript text. C.T., W.A. and S.S. analysed the main data and made the interpretation of the data. All authors reviewed the manuscript.

Competing Interests

The authors declare no competing interests.

Conflict of Interest

declare no conflict of interest

An Ethical Statement

This study was a case-control study conducted at the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Ethical approval was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. IRB No. 577/59

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