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Original Article

Upregulation of microRNA-223 expression in gingival crevicular blood of women with gestational diabetes mellitus and periodontitis

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Background/Purpose: MicroRNA-223 (miR-223) is involved in several inflammatory diseases, including gestational diabetes mellitus (GDM) and periodontitis. We first described a procedure for purifying miR-223 from gingival crevicular blood (GCB) of pregnant women with or without GDM and periodontitis. This study aimed to determine whether GDM and/or periodontitis modifies miR-223 expression in pregnant women and to analyze miR-223-targeted messenger RNA (mRNA) expression levels in GCB compared to peripheral blood (PB).
Materials and methods: Pregnant women were allocated to 4 groups: 10 women with GDM and periodontitis (GDM/P), 10 women with GDM without periodontitis (GDM/NP), 9 women with periodontitis and without GDM (NGDM/P) and 10 women without either condition (NGDM/NP). Clinical parameters of GDM and periodontal status were examined. GCB and PB were collected to assess miR-223, ICAM-1, IL-1β and β1-integrin gene expression by quantitative real-time polymerase chain reaction.
Results: The GDM/P group demonstrated the highest miR-223 expression levels among the 4 groups in GCB. A significant difference was found between GDM/P and GDM/NP group ($P = 0.04$). In contrast, the GDM/P showed the lowest miR-223 expression level in PB among

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the 4 groups. Moreover, ICAM-1 and IL-1β mRNA expression exhibited the opposite trend of miRNA-223, indicating that miRNA-223 might regulate the mRNA function of those genes by epigenetic events.
Conclusion: The upregulation of miR-223 expression in GCB but downregulation in PB, ICAM-1 and IL-1β genes expression in women with GDM and periodontitis suggest a promising role of miR-223 in the association between GDM and periodontitis.

Introduction

Gestational diabetes mellitus (GDM) is any degree of glucose intolerance with onset during pregnancy.[1] This condition is associated with long-term maternal and fetal health risks, including type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD).[2] Similar to T2DM, several inflammatory mediators, e.g., c-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6),[3] IL-1β,[4] and intercellular adhesion molecule-1 (ICAM-1),[5] have been linked to the insulin resistance and beta-cell dysfunction observed in women with GDM.[6]

Periodontitis is characterized by chronic inflammation of the tissues surrounding the teeth.[7] This condition may contribute to low-grade systemic inflammation and has been associated with inflammatory systemic diseases.[8,9] Given the similarity in the pathogenesis of T2DM and GDM, these conditions may be associated, however the evidence is still inconclusive.[3,10,11]

Recently, interest in the role of small noncoding RNAs (20-22 bases) called microRNAs (miRNAs) has increased. The primary role of miRNAs is the regulation of messenger RNA (mRNA) function by specific binding to target mRNAs, which mainly results in the degradation of the mRNA and inhibition of protein translation by epigenetic events.[12] Among the substantial number of newly identified miRNAs, miR-223 has been reported to be linked to T2DM,[13] GDM[14] and periodontitis.[15] Previous studies consistently reported that aberrant miR-223 expression is related to glucose metabolism[13,16–18] and several processes involved in periodontitis.[15,19–22] Additionally, ICAM-1,[23] IL-1β[24] and β1-integrin,[25] the biomarkers potentially involved in GDM and periodontitis, were reported to be targeted by miR-223.

In the present study, we performed a novel collection method of gingival crevicular blood (GCB), which might be enriched with miRNAs from both periodontal cells and the contents in circulation, such as platelets, and might reflect the differential pattern of miR-223 expression modulated by periodontitis and GDM. Thus, the objectives of the present study were to compare the level of miR-223 expression isolated from GCB among pregnant women who had GDM with/or without periodontitis, to investigate the level of miR-223 expression in peripheral blood (PB) among these groups and to analyze the effects of miR-223 on the mRNA expression of ICAM-1, IL-1β and β1-integrin.

Materials and methods

Study design and participant recruitment

This cross-sectional pilot study was approved by the Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (COA. No. Si 107/2019). Forty pregnant women were consecutively recruited from the antenatal care clinic, Faculty of Medicine Siriraj Hospital, from May 2019 to December 2019. The inclusion criteria were as follows: 1) gestational age $\leq$ 28 weeks (early 3rd trimester) and 2) women who had undergone the screening and diagnostic procedure for GDM. In Siriraj Hospital, GDM is routinely screened and diagnosed during the first visit for antenatal care and at 28–32 weeks of gestation by a two-step approach using a glucose challenge test (GCT) followed by a 100-g oral glucose tolerance test (OGTT).[26] Potential participants were excluded if they met any of the following criteria: 1) presence of diseases that affect periodontal conditions; 2) presence of serious systemic conditions, including heart disease, hemophilia, thrombocytopenia and HIV positivity; 3) periodontal therapy 6 months prior to the day of enrollment; 4) use of insulin or antidiabetic drugs; 5) need for antibiotic prophylaxis prior to dental procedures; 6) current smoking and alcohol drinking habit; and 7) number of teeth less than 20 excluding third molars. Data regarding maternal age, gestational age, body mass index (BMI), 50-g GCT and the GDM screening and diagnostic test results of the potential participants who agreed to participate in the study and signed the written informed consent form were collected from antenatal care record forms by experienced study personnel.

At the enrollment visit, potential participants were subjected to a full-mouth periodontal examination by one dentist blinded to the GDM status. The following clinical parameters were assessed using a standard manual probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA) at mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual sites of each tooth excluding the third molar: pocket depth (PD), clinical attachment level (CAL), dental plaque (Pl) and bleeding on probing (BOP). Probing depth was measured to the nearest millimeter from the gingival margin (GM) to the bottom of the pocket. CAL was calculated as the arithmetic summation of probing depth and the distance from the GM to the CEJ at each site. Plaque was designated as present if it was detected by the naked eye

or by running the tip of a periodontal probe along the tooth surface at the GM of each site. BOP was defined as present if bleeding occurred within 20 s after periodontal probing. % PI and %BOP were then calculated from the percentage of positive sites by the total number of sites per mouth. The criteria proposed by Offenbacher et al.[27] were used in this study to diagnose periodontitis. Participants with periodontitis (P) were those who had moderate-to-severe periodontitis (at least 4 sites with PD $\geq$ 5 mm and 4 sites with CAL $\geq$ 2 mm). Participants with no periodontitis (NP) referred to those who had less disease than the moderate-to-severe group or those who had good periodontal health (absence of any PD $>$ 3 mm and no sites with CAL $>$2 mm). Therefore, participants were enrolled in four groups (ten/group) including GDM/P, GDM/NP, NGDM/P and NGDM/NP.

Sample collection and preparation

The supragingival plaque was removed using a sterile curette at the selected sites, which were visually red with BOP and PD $\geq$ 5 mm for the P group and $<$5 mm for the NP group; then, root planing was performed at the selected site, followed by careful insertion of a micropipette tip into the periodontal pocket. Approximately 200–500 μL of GCB was drawn from the gingival sulcus by using a micropipette with a capillary piston tip (MICROMAN E, Gilson, Middleton, WI, USA), transferred to 0.5 mL EDTA microtubes (Hebei Xinle Sci&Tech, Xinle, China) and stored at −80 °C in a freezer. Moreover, approximately 1 mL of PB was collected, drawn into 3 mL EDTA vacuum tubes and immediately stored at −80 °C for further analysis of miR-223 and mRNA expression.

Total RNA extraction

Total RNA was isolated from GCB and PB using a kit according to the manufacturer's recommendation (miRNeasy Mini kit, Qiagen, Germantown, MD, USA). Then, RNA purity and concentration were evaluated by spectrophotometry (NanoDrop 2000/2000c, Thermo-Fisher Scientific, Fremont, CA, USA).

Quantitative real-time polymerase chain reaction

Approximately 1 μg of RNA was reverse transcribed to cDNA using a miRNA reverse transcription kit (miScript II RT kit Qiagen, Hilden, Germany). The miR-223 expression level was determined using SYBR green for real-time polymerase chain reaction (PCR) (miScript SYBR Green PCR kit, Qiagen, Hilden, Germany) according to the manufacturer's protocols. To determine the mRNA level, we used a qPCR kit based on the manufacturer's instructions (KAPA SYBR® FAST Master Mix (2X) kit, Kapa Biosystems, Cape Town, South Africa). All gene validation experiments were performed in duplicate.

We analyzed the relative expression rates of miR-223 in the control (NGDM/NP) and the test groups with internal controls (miR-16 and U6) using the method proposed by Vandesompele et al.[28] The 2ΔΔCT method was used for mRNA analysis. Delta Ct (ΔCt) values for each mRNA were obtained after normalization to β-actin. The relative mRNA expression of each sample was calculated by 2ΔΔCT, where ΔΔCt = ΔCt (diseased group) − ΔCt (control group). The results are reported as the fold change in the relative expression level. The sequences of the PCR primers used are listed in Table 1.[29,30]

Statistical analyses

The normality of the data was analyzed by the Shapiro–Wilk test. The descriptive data were presented as the mean value ± standard deviation (SD) or as the mean ± standard error of the mean (SEM) for gene expression validation. For multiple-group comparisons, one-way ANOVA and Tukey's post hoc test were used to test variables with a normal distribution. The Kruskal–Wallis test with the Dunn-Bonferroni post hoc test was used for gestational age, BMI, 50-g GCT, %site with PD $\geq$ 5 mm, %site with CAL $\geq$ 2 mm, and expression of miR-223, ICAM-1 and IL-1β in PB. The intragroup comparison of miR-223 between GCB and PB was performed with the Wilcoxon signed rank test. In addition, the correlation coefficient determined using Pearson and Spearman's rank was applied to investigate the correlation between gene expression (miR-223, ICAM-1, IL-1β and β1-integrin) and the other clinical variables. We considered $P < 0.05$ to indicate a significant difference.

Results

Demographic characteristics and clinical periodontal parameters of the study participants

The demographic characteristics and clinical periodontal parameters of the study population for the four study groups were presented in Table 2. The GDM/P group exhibited the highest levels of all periodontal variables and significantly higher %PI, %BOP, mean PD and mean CAL values than the GDM/NP and NGDM/NP groups. Moreover, the GDM/P and NGDM/P groups had significantly higher values of %site with PD $\geq$ 5 mm than the GDM/NP and NGDM/NP groups ($P < 0.001$). Data were shown as the mean (standard deviation).

MiR-223 expression analysis

The relative expression of miR-223 in GCB of the GDM/P group was the highest among the 4 groups, and a significant difference was found between the GDM/P and GDM/NP groups ($P = 0.04$) (Fig. 1a). In contrast to that in GCB, miR-223 expression in the PB of the GDM/P and GDM/NP groups was visibly down regulated relative to that in the NGDM/P and NGDM/NP groups. The difference in miR-223 levels in PB was not significant among the four groups ($P = 0.082$). When an intragroup comparison of miR-223 expression levels between GCB and PB in related subjects was performed, a trend of difference between GCB and PB was found within the GDM/P and GDM/NP groups ($P = 0.109$) (Fig. 1b). The miR-223 expression in GCB had negative

Table 1 Nucleotide sequences of primers for miRNAs and mRNAs.

	Gene		5′-3′ primer sequence,	Genbank
miRNAs	miR-223	FW	TGTCAGTTTGTCAAATACCCCA	AJ550427.1
	miR-16	FW	GTAGCAGCACGTAAATATTGG	Zhang et al.[29]
	U6	FW	GCAAGGATGACACGCAAATTC	Zhang et al.[30]
mRNAs	ICAM-1	FW	GGCTGGAGCTGTTTGAGAAC	NM_000201.3
		RT	ACTGTGGGGTTCAACCTCTG	
	IL-1β	FW	GGGCCTCAAGGAAAAGAATC	NM_000576.3
		RT	TTCTGCTTGAGAGGTGCTGA	
	β1-integrin	FW	AATGAATGCCAAATGGGACACGGG	NM_002211.3
		RT	TTCAGTGTTGTGGGATTTGCACGG	
	β-actin	FW	AGAGCTACGAGCTGCCTGAC	NM_001101.3
		RT	AGCACTGTGTTGGCGTACAG	

Table 2 Demographic characteristics and clinical periodontal parameters of the study subjects.

	GDM/P (n = 10)	GDM/NP (n = 10)	NGDM/P (n = 9)	NGDM/NP (n = 10)	*P* value
Age (years)	33.7 (6.29)	31.3 (4.72)	32.78 (4.87)	30.3 (4.57)	0.475
Gestational age (weeks)	22 (5.35)	20.2 (6.48)	22.33 (3.50)	24.3 (3.77)	0.477
BMI (kg/m^2)	25.9 (5.14)	25.69 (5.41)	21.9 (3.85)	25.86 (6.00)	0.218
50 g-GCT (mg/dL)	195 (35.52)[a]	213.8 (21.44)[a,b]	125.89 (29.05)	139.30 (43.37)	<0.001
%PI	95.14 (3.83)[b,c]	84.50 (6.87)	86.77 (7.09)	83.13 (10.91)	0.001
%BOP	82.12 (11.59)[b,c]	62.64 (10.54)	72.17 (14.45)	59.32 (19.51)	0.005
Mean PD	3.15 (0.27)[b,c]	2.57 (0.25)	2.95 (0.23)	2.69 (0.22)	<0.001
Mean CAL	3.00 (0.67)[b,c]	2.21 (0.27)	2.55 (0.56)	2.22 (0.37)	0.018
%site with PD ≥ 5 mm	5.37 (5.05)[b,c]	0.25 (0.60)	4.15 (2.85)[b,c]	0.07 (0.21)	<0.001
%site with CAL ≥ 2 mm	90.96 (15.58)[b,c]	85.63 (9.14)	86.78 (16.46)[b,c]	81.91 (21.64)	0.223

Data was shown as mean (standard deviation). [a]$P < 0.05$ compared with the NGDM/P group. [b]$P < 0.05$ compared with the NGDM/NP group. [c]$P < 0.05$ compared with the GDM/NP group. GDM/P: gestational diabetes mellitus with periodontitis, GDM/NP: gestational diabetes mellitus without periodontitis, NGDM/P: systemically healthy with periodontitis, NGDM/NP: systemically healthy without periodontitis.

correlation with miR-223 expression in PB, but no significant difference was found (r = −0.086, $P = 0.77$, Fig. 1b).

ICAM-1, IL-1β and β1-integrin mRNA expression analyses

According to Fig. 2, the GDM/P group showed higher relative expression of all mRNAs than the other groups, except for β1-integrin in PB. However, a significant difference was found only in the comparison of IL-1β expression in PB, in which the GDM/P group exhibited a significantly higher level than the GDM/NP group (3.12 vs 0.14, $P < 0.05$).

The correlation between miR-223 expression and periodontal clinical variables

As shown in Table 3, miR-223 expression in GCB was positively correlated with periodontal parameters, including % PI, %BOP, and mean PD (r = 0.55, $P < 0.001$, r = 0.55, $P < 0.001$, and r = 0.43, $P = 0.006$, respectively). In contrast, miR-223 expression in PB was negative correlated with %PI, mean PD, and mean CAL, but there was no statistically significant difference ($P > 0.05$).

The correlation between miR-223 expression and targeted mRNA expression

For clarify the relationship between miR-223 and targeted mRNA expression, including ICAM-1, IL-1β and β1-integrin. MiR-223 in GCB demonstrated negative correlation to IL-1β and β1-integrin in GCB. MiR-223 in PB also demonstrated negative correlation to IL-1β and β1-integrin in both GCB and PB. However, all correlation was not significantly different ($P > 0.05$, Table 4).

Discussion

We first examined miR-223 expression in GCB among pregnant women who had GDM with/or without periodontitis. The significant overexpression of miR-223 in GCB from GDM/P group may be influenced by GDM combined with periodontitis. Furthermore, the result demonstrating that GCB miR-223 expression was positively correlated with clinical periodontal parameters might suggest that elevated GCB miR-223 levels in the periodontitis groups was primarily linked to the inflammatory response at periodontitis sites. This finding was comparable to previous reports showing that an increased miR-223 expression was

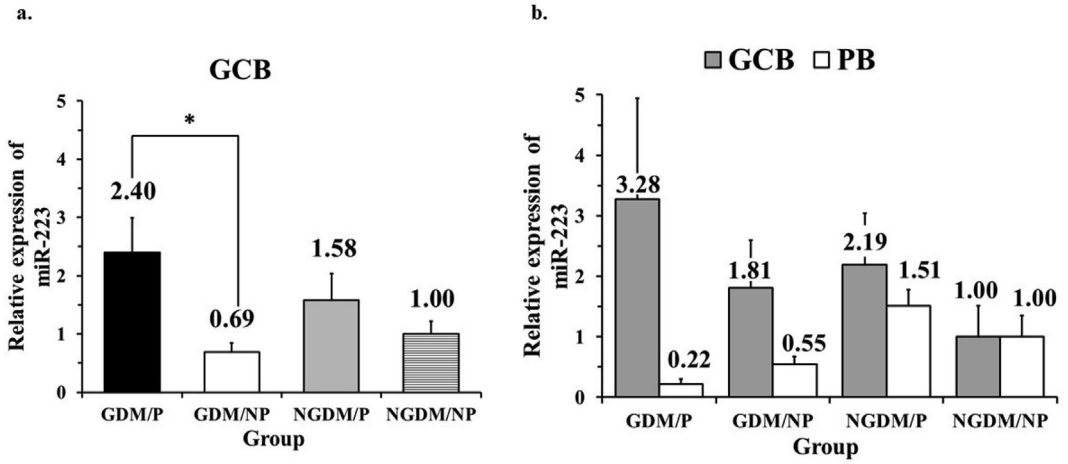

Figure 1 Relative expression level of miR-223 in GCB from all subjects in each of the study groups. *$P < 0.05$, determined by one-way ANOVA with Turkey Multiple Comparison post-test (a). Intragroup comparison between the relative expression level of miR-223 in the GCB and the PB was tested by Wilcoxon Signed Rank test (b). Values are indicated with error bars as mean ± SEM. GCB: gingival crevicular blood, PB: peripheral blood, GDM/P: gestational diabetes mellitus with periodontitis, GDM/NP: gestational diabetes mellitus without periodontitis, NGDM/P: systemically healthy with periodontitis, NGDM/NP: systemically healthy without periodontitis.

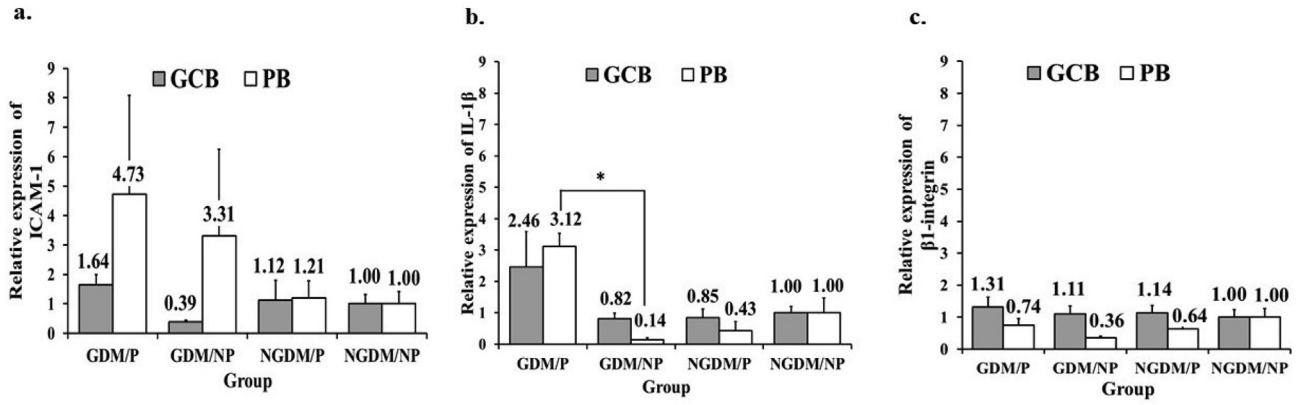

Figure 2 Comparison of mRNA (ICAM-1, IL-1β, β1-integrin) expression level in GCB and PB (a–c) among the four groups. Values are indicated with error bars as mean ± SEM. *$P < 0.05$. GCB: gingival crevicular blood, PB: peripheral blood, GDM/P: gestational diabetes mellitus with periodontitis, GDM/NP: gestational diabetes mellitus without periodontitis, NGDM/P: systemically healthy with periodontitis, NGDM/NP: systemically healthy without periodontitis.

Table 3 Correlation between miR-223 expression and clinical variables.

		%PI	%BOP	Mean PD	Mean CAL
GCB	*r*	0.55	0.55	0.43	0.11
miR-223	*P*	<0.001	<0.001	0.006	0.512
PB	*r*	−0.50	0.10	−0.10	−0.17
miR-223	*P*	0.072	0.728	0.723	0.560

GCB: gingival crevicular blood, PB: peripheral blood.

consistently identified in gingival tissue and GCF from sites with periodontal disease compared with healthy sites.[15,19,31] In neutrophils, miR-223 functions as a negative regulator of the inflammatory response by regulating transcription factors such as NLRP3[24] and also directly targeting of the chemoattractants CXCL2, CCL3 and IL-6.[32] This finding was supported by animal studies demonstrating that miR-223-deficient mice had neutrophils with a hyperactive phenotype.[33] In contrast, miR-223 elevation in human gingival fibroblasts and monocytes was found to facilitate the production of proinflammatory cytokines and the differentiation of osteoclasts, respectively.[21,22] In this context, these results showed that miR-223 functions in a cell-specific manner, so its upregulated expression in the present study could not be specifically considered as a protective or pathogenic role in periodontal pathogenesis. However, the comparable pattern of miR-223 overexpression among GCB, GCF and biopsy gingival tissue suggested that collection of GCB in the present study might be a suitable source for miRNA studies.

Interestingly, the expression pattern of miR-223 in PB was decreased and showed the opposite trend of the GCB.

Table 4 Correlation between miR-223 expression and targeted mRNAs expression.

		ICAM-1 GCB	IL-1β GCB	β1-integrin GCB	ICAM-1 PB	IL-1β PB	β1-integrin PB
GCB	*r*	0.37	−0.14	−0.15	0.34	0.29	−0.05
miR-223	*P*	0.117	0.576	0.531	0.158	0.214	0.840
PB	*r*	0.29	−0.50	−0.67	−0.24	−0.55	0.21
miR-223	*P*	0.493	0.253	0.069	0.570	0.160	0.610

GCB: gingival crevicular blood, PB: peripheral blood.

The lower miR-223 expression in PB from the GDM groups than in PB from the NGDM groups was consistent with previous reports performed in populations with T2DM.[13,16] The evidence illustrated that platelet could be suppressed by hyperglycemia, which in turn resulted in platelet function activation via deregulated expression of the P2Y12 receptor.[16] As miRNAs are expressed in a tissue-specific manner, blood samples received from different sources might contain different types and characteristics of miRNAs. GCB contains miRNAs from blood vessels localized in gingival connective tissue surrounded by abundant periodontal resident cells. MiR-223 was shown to be highly expressed in neutrophils and macrophages and could be secreted into the extracellular matrix and blood vessels in a microvesicle form.[20] In contrast, platelet in PB is believed to be a major source of miR-223 production.[34] Therefore, overexpression of miR-223 in GCB from the GDM/P group might reflect localized periodontal inflammation rather than systemic glucose elevation in GDM.

In addition, the trend of higher mRNA levels of IL-1β and ICAM-1 in both GCB and PB from the GDM/P group might indicate the synergistic effect of periodontal inflammation and GDM on the systemic response. These results suggested that inflammatory mediators triggered by periodontal disease might aggravate pregnancy-induced insulin resistance and impaired the glucose tolerance observed in GDM.[3] Besides, the significant difference of β1-integrin expression, a molecule which had a role in angiogenesis in the vascular endothelium and severe diabetic complications,[25,35] was not found in this study possibly due to a short GDM duration.

There were some limitations in present study. First, the sample size might be too small to reach statistical significance. Second, different periods of time from GDM diagnosis to the recruitment date of each participant might affect gene expression due to a varied capability of glycemic control. Third, the underlying GDM risk factors, such as hypertension and family history of diabetes, which might affect miR-223 expression, were not included in the analysis.

In conclusion, women with GDM and periodontitis exhibited upregulation of miR-223 expression in GCB but downregulated expression in PB. The aberrant expression of miR-223 in this group might be related to the elevation of miR-223-targeted proinflammatory cytokines. This result suggested that overexpression of miR-223 mainly contributed to periodontitis and was possibly modified by GDM. GCB might be a novel source of biomarkers for further studies to identify other miRNAs related to GDM and periodontitis. In the future, the collection of GCB might be clinically applied in routine screening programs as a diagnostic tool for the early detection or prevention of GDM and periodontitis in pregnant women.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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