Original Paper

MicroRNA-873 is a Potential Serum Biomarker for the Detection of Ectopic Pregnancy

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Key Words

Ectopic pregnancy • Biomarker • MicroRNA-873 • Diagnosis

Abstract

Background: Ectopic pregnancy (EP) refers to the implantation of the zygote outside the uterine cavity. In clinical practice, the diagnosis of EP relies on a combination of ultrasound findings and serum human chorionic gonadotrophin (hCG) measurements. However, the need for serial hCG measurements increases the risk of tubal rupture and death, underscoring the need to identify biomarkers for the early detection of EP. **Methods:** The serum concentrations of 21 microRNAs (miRNAs) associated with pregnancy or with known placental expression, as well as serum hCG and progesterone levels were analyzed 36 patients with viable intrauterine pregnancy (VIP), 30 patients with spontaneous abortion (SA), and 34 patients with EP using specific assay kits and reverse transcription PCR. The diagnostic performance of the different serum markers for detecting EP was analyzed by ROC curve analysis. **Results:** Five miRNAs were differentially expressed between the three groups, of which miR-873 and miR-223 were significantly lower in EP than in VIP and SA patients and did not change significantly according to gestational age, and miR-323 was significantly higher in EP than in VIP and SA. As a single marker, miR-873 had the highest sensitivity for detecting EP at 61.76% (at a fixed specificity of 90%). In comparison, the combination of hCG, progesterone and miR-873 had the highest sensitivity for detecting EP at 79.41% (at a fixed specificity of 90%). **Conclusion:** Although further validation in large-scale prospective studies is necessary, our results suggest that miR-873 could be a valuable noninvasive and stable biomarker for the early detection of EP.

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Introduction

Ectopic pregnancy (EP) is a condition in which the fertilized egg implants in a location other than the inner lining of the uterus [1]. The incidence of EP has increased markedly

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in the last decades from 4.5 per 1000 pregnancies in 1970 to an estimated 19.7 per 100 pregnancies in 1992, and it remains an important cause of maternal death, accounting for approximately 9–13% of pregnancy-associated deaths in developed countries and 10–30% in African developing countries [2, 3]. EP is diagnosed by transvaginal ultrasonography, which detects an extrauterine gestational sac containing a yolk sac; however, additional assessment by surgical or biochemical methods is necessary in approximately 8–31% of patients [4]. The most common biochemical markers for the diagnosis of EP are serum human chorionic gonadotropin (hCG) and progesterone. Progesterone is an indicator of fetal viability and concentrations above 25 ng/ml indicate a viable intrauterine pregnancy (VIP), whereas concentrations below 5 ng/ml are non-viable [5]. For hCG, serial monitoring every 48 h is necessary for a positive diagnosis of EP; an increase in hCG of $\geq 66\%$ in 48 h is associated with VIP, whereas an increase of $< 50\%$ is associated with abnormal pregnancy including EP [6, 7]. However, these measurements cannot identify the location of the gestational sac and are associated with high false-positive and false-negative rates, in addition to putting the patient at risk of tubal rupture in between measurements [8]. Furthermore, these markers are not effective for distinguishing between EP and spontaneous abortion [9]. Therefore, there is a need to develop accurate and sensitive noninvasive tests for the diagnosis of EP.

MicroRNAs (miRNAs) are small (22 nucleotides) endogenous single-stranded RNAs that regulate gene expression by binding to the 3' untranslated region of target genes, leading to translational repression or degradation of the targeted transcript [10]. MiRNAs are aberrantly expressed in many diseases, and their stability and resistance to degradation makes them useful biomarkers in cancer, autoimmune diseases, sepsis, and acute myocardial infarction among others [11]. Pregnancy-associated circulating miRNAs have been researched extensively in recent years as potential biomarkers for the diagnosis of pregnancy-associated complications, and alterations in the concentration of these miRNAs has been shown to reflect pathological states such as preeclampsia, preterm labor, and fetal growth restriction among others [12–16].

In the present study, we examined the serum concentration of different miRNAs to identify pregnancy-associated miRNAs differentially expressed in patients with EP, SA, and VIP and analyzed their diagnostic accuracy as biomarkers for the detection of EP in an emergency department (ED) setting.

Materials and Methods

Specimen collection

Patients were recruited from the International Peace Maternity and Child Health Hospital and JiangWan Hospital, Shanghai. All patients included agreed to participate in the study, and the study was approved by Ethical Board of Shanghai Jiao Tong University School of Medicine. Remnant serum samples sent to the laboratory from the ED for physician-ordered hCG testing between 2013 and 2016 were included in the analysis. All serum samples sent to the laboratory for quantitative hCG testing were retained and frozen at -70°C before progesterone and miRNA testing was performed.

HCG and progesterone testing

All hCG and progesterone measurements were performed at International Peace Maternity and Child Health Hospital and JiangWan Hospital using commercially available assays. Serum hCG and serum progesterone were assayed using the Centaur Total hCG assay and the Centaur Progesterone assay, respectively.

Serum Isolation and RNA extraction

Total RNA containing miRNAs was extracted from 250 μL serum using the TRIzol LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol with modifications.

Primers

All primers were synthesized and purchased from Integrated DNA Technologies (IDT, Coralville, IA), and the primers of candidate miRNAs are listed in Table 1.

Table 1. The sequences for the primers. ^aThe sequences that are in common are in bold font and the sequences that are miRNA specific are in italic fonts. ^bUniversal Reverse primer

Target	stem-loop Reverse primer ^a	forward primer ^a
hsa-mir-196b	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGAAGGCAG	ACACTCCAGCTGGGTTCGACAGCAGCAGACT
hsa-mir-30a	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGCAGCTGC	ACACTCCAGCTGGGCTTTTCAGTCGGATGTTTG
hsa-mir-873	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTCCCGGGA	ACACTCCAGCTGGGGGAGACTGATGAGT
hsa-mir-337-3p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGAAGAAAG	ACACTCCAGCTGGGCTCCTATATGATGC
hsa-mir-1288	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTCTCCAGA	ACACTCCAGCTGGGTGGACTGCCCTGA
hsa-mir-451	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAACTCAGT	ACACTCCAGCTGGGAAACCGTTACCAT
hsa-mir-223	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTGGGGTAT	ACACTCCAGCTGGGTGTCAGTTTGTCAA
hsa-mir-141	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCCATCTTT	ACACTCCAGCTGGGTAACTGTCTGGTAA
hsa-mir-151-5p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACTAGACT	ACACTCCAGCTGGGTTCGAGGAGCTCACAG
hsa-mir-520a-5p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGAAAGTA	ACACTCCAGCTGGGCTCCAGAGGGAAGTA
hsa-mir-525-3p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCGCTCTAA	ACACTCCAGCTGGGGAAGGCGCTTCCCTT
hsa-mir-433	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACACCGAG	ACACTCCAGCTGGGATCATGATGGGCTCCT
hsa-mir-487a	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAACTGGAT	ACACTCCAGCTGGGAATCATACAGGGACAT
hsa-mir-218	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACATGGTT	ACACTCCAGCTGGGTGTGCTTGATCTAA
hsa-mir-204	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGGCATAG	ACACTCCAGCTGGGTTCCTTTTGTCATCC
hsa-mir-516-5p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAAAGTGCT	ACACTCCAGCTGGGATCTGGAGGTAAGAG
hsa-mir-517	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACACTCTA	ACACTCCAGCTGGGATCGTGATCCCTT
hsa-mir-518b	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACCTCTAA	ACACTCCAGCTGGGCAAGCGCTCCCTTT
hsa-mir-526a	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGCAGAAAGT	ACACTCCAGCTGGGCTCTAGAGGGAAGCAC
hsa-mir-154	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAAATAGGTC	ACACTCCAGCTGGGAATCATACAGGTTGA
hsa-mir-323-3p	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAGAGGTCG	ACACTCCAGCTGGGCACATTACACGGTGC
universalR ^b		TGGTGTCTGGAGTCTG
18s	CACAGTTATCCAAGTGGGAGAGG	GAAACTGCGAATGGCTCATTA

Reverse transcriptase reactions

For each reaction, 4 μ L RNA, 5 nmol/L of each miRNA-specific reverse stem-loop primer, 1 $\times$ RT buffer, 0.25 mM each of dNTPs, 1 μ L MultiScribe reverse transcriptase and 1 μ L RNase inhibitor were added into a 10 μ L multiplex reverse transcription reaction (High-Capacity cDNA Archive Kit; Applied Biosystems). Reactions were incubated in a 96-well plate for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C.

Real-time quantitative reverse-transcription (RT) PCR analysis

The 10 μ L multiplex PCR reaction included 0.5 μ L RT product, 1 $\times$ TaqMan Universal PCR Master PCR Mix, 1 μ mol/L of the universal reverse primer, 1 μ mol/L each miRNA forward primer and 0.1 μ mol/L of TaqMan probe. PCR reactions were analyzed on an Applied Biosystems 7500 Fast Real-Time PCR System. PCR conditions were 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Ct values were calculated and converted into fold-change relative to that of 18s RNA.

Statistical analysis

Patient age, gestational age (GA), and concentrations of serum hCG and progesterone, and miRNAs of the EP, SA, and VIP patients were compared. Because the distributions of these variables were not Gaussian, the Kruskal-Wallis test and the Dunn multiple-comparison post hoc test were used to determine statistical significance. ROC curve analysis was performed, and areas under the curve (AUCs) were calculated. Sensitivity and specificity were calculated using Pearson χ^2 analysis. The cutoff point of each step was determined by fixing the sensitivity at the highest level. The ANOVA test, box plot were performed with GraphPad Prism (version 5.0; GraphPad Software), and ROC analysis was performed with SPSS (version 17.0; SPSS Inc., Chicago, IL). Statistical significance was defined as a P value <0.05.

Results

Serum concentrations of hCG, progesterone, miR-218, miR-223, miR-141, miR-873 and miR-323 in patients with EP, SA, and VIP

A total of 36 patients with VIP, 34 patients with EP, and 30 patients with SA were included in the analysis, and the median (range) ages were 28 (25–31.5), 30 (27–34), and 28.5 (25–33) years, respectively. There were no significant differences in age or GA between the three groups. Patient characteristics and serum values of hCG, progesterone, and specific miRNAs in patients with EP, SA, and VIP are shown in Table 2. Serum hCG and progesterone

Fig. 1. Expression of hCG, progesterone and five miRNAs in EP, SA and VIP patients. (A-G) Box plots of measurements of hCG, progesterone, miR-218, miR-223, miR-141, miR-873 and miR-323 in patients with EP, SA, and VIP. The median, interquartile range, and the data range are shown; outliers are also indicated. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Dunn multiple-comparison test.

concentrations were significantly lower in patients with EP and SA than in those with VIP ($P < 0.01$, Fig. 1A and 1B), and lower in EP than in SA, although the difference did not reach statistical significance. A total of 21 miRNA candidates (hsa-mir-196b, hsa-mir-30a, hsa-mir-873, hsa-mir-337-3p, hsa-mir-1288, hsa-mir-451, hsa-mir-223, hsa-mir-141, hsa-mir-151-5p, hsa-mir-520a-5p, hsa-mir-525-3p, hsa-mir-433, hsa-mir-487a, hsa-mir-218, hsa-mir-204, hsa-mir-516-5p, hsa-mir-517, hsa-mir-518b, hsa-mir-526a, hsa-mir-154 and hsa-mir-323-3p) with a known association with pregnancy or placental specificity were analyzed to identify potential candidate miRNAs capable of distinguishing between women with EP, VIP, and SA. We identified miR-218, miR-223, miR-873, miR-141 and miR-323 as differentially expressed between the three groups. The serum concentration of miR-218 was higher in EP and VIP than in SA ($P < 0.05$ both, Fig. 1C), but did not differ significantly between EP and VIP; miR-223 was lower in VIP than in EP and SA ($P < 0.001$, Fig. 1D) but did not differ significantly between EP and VIP; miR-141 was higher in EP and SA than in VIP ($P < 0.01$ and $P < 0.001$, Fig. 1E) but did not differ significantly between EP and SA; miR-873 was lower in EP than in SA and VIP ($P < 0.001$ both, Fig. 1F); however, miR-323 was significantly higher in EP than in VIP and SA ($P < 0.01$ both, Fig. 1G) (Table 2). Analysis of the correlations between the serum concentrations of hCG and progesterone and GA in the different groups showed that hCG was significantly positively correlated with GA in the VIP group (Fig. 2A), and progesterone concentration was negatively correlated with GA in the VIP group, but not in the EP or SA groups (Fig. 2B). MiR-218 expression was positively correlated with GA only in the VIP group (Fig. 2C). However, miR-223, miR-141, miR-873 and miR-323 concentrations in the EP, SA, and VIP groups showed no correlation with GA.

Diagnostic performance of serum hCG, progesterone, and selected miRNAs, and in combination

The diagnostic performance of the different serum markers for detecting EP was analyzed by ROC curve analysis in a cohort of 34 EP patients and 66 non-EP controls (Table 3, Fig. 3A). The sensitivities at the predefined specificities of 90% and 95% and the specificities at the predefined sensitivities of 90% and 95% were calculated for all markers

Table 2. Patient age, GA, and serum concentrations of hCG, progesterone, and miRNAs in women with EP, SA, or VIP. ^aData are expressed as the median (interquartile range). ^bDunn multiple-comparison post hoc test. ^cNS, not statistically significant ($P \geq 0.05$). ^dmiRNA concentrations are expressed relative to 18S rRNA

	VIP (n=36) ^a	EP (n=34) ^a	SA (n=30) ^a	Kruskal-Wallis test	EP vs SA ^b	EP vs VIP ^b	SA vs VIP ^b
Patient age, years	28 (25-31.5)	30 (27-34)	28.5 (25-33)	NS ^c	NS	NS	NS
GA, weeks	7.79 (5.75-8.57)	6.86 (5.97-7.97)	7.00 (6.12-9.12)	NS	NS	NS	NS
hCG, IU/L	56695 (10161-85715)	1550 (489.9-4977)	3148 (1007-4913)	< 0.001	NS	$p < 0.001$	$p < 0.001$
Progesterone, ng/ml	28.55 (22.36-34.95)	7.85 (5.46-11.43)	12.17 (5.95-16.85)	< 0.001	NS	$p < 0.001$	$p < 0.001$
miR-218 ^d	0.95 (0.23-2.83)	1.25 (0.28-3.34)	0.16 (0.064-1.06)	< 0.01	$p < 0.05$	NS	$p < 0.05$
miR-223 ^d	0.33 (0.14-0.45)	0.44 (0.19-0.72)	1.64 (0.65-2.62)	< 0.001	$p < 0.001$	NS	$p < 0.001$
miR-873 ^d	5.10 (2.65-6.43)	0.089 (0.031-2.28)	3.79 (2.14-6.07)	< 0.001	$p < 0.001$	$p < 0.001$	NS
miR-141 ^d	0.090 (0.027-0.26)	0.68 (0.25-1.05)	0.49 (0.17-1.63)	< 0.001	NS	$p < 0.01$	$p < 0.001$
MiR-323 ^d	0.61 (0.11-2.52)	2.45 (1.16-2.02)	0.47 (0.097-2.45)	< 0.001	$p < 0.01$	$p < 0.01$	NS

Fig. 2. Expression of hCG, progesterone and five miRNAs in different weeks of gestational age (GA). (A-G) Serum concentrations of hCG, progesterone, miR-218, miR-223, miR-141, miR-873, and miR-323 according to GA at the time of emergency department visit in patients with EP, SA, and VIP.

individually and in combination as shown in Table 3. As single markers, the AUC values were as follows: progesterone vs. hCG: 0.82 [95% confidence interval (CI) 0.73-0.91] vs. 0.74 (95% CI 0.65-0.84); miR-323 and miR-873: 0.74 (95% CI 0.64-0.84) and 0.84 (95% CI 0.76-0.92), respectively. MiR-323 had a higher sensitivity for the detection of EP than hCG and progesterone at the fixed specificities of 90% and 95% (47.60% and 32.35%, respectively). However, miR-873 had the highest sensitivity for the detection of EP as a single marker at the fixed specificities of 90% and 95% (61.76% and 55.88%, respectively). To determine the value of the miRNAs as biomarkers, we conducted a multivariate logistic regression analysis to compare the different combinations of biomarkers with that of the dual markers of hCG plus progesterone as a standard reference. The AUCs of different multimarker combinations were 0.84 to 0.92 (Table 3, Fig. 3B). The combinations of hCG, progesterone and miR-323 produced higher sensitivities at 67.65% and 50.00% than those of hCG plus progesterone at 55.85% and 44.12% with specificities of 90% and 95%, respectively (both $P < 0.05$). The combinations of hCG, progesterone and miR-873 produced the highest sensitivities of 79.41% and 64.71% compared with 55.85% and 44.12% for hCG plus progesterone with specificities of 90% and 95%, respectively (both $P < 0.01$). The combinations of hCG, progesterone, miR-873 and miR-323 produced higher sensitivities at 70.59% and 52.94% than 55.88% and

Table 3. Diagnostic accuracies of measurements of single markers and of multimarker combinations for predicting EP in patients with abdominal pain and/or vaginal bleeding at predefined specificities and sensitivities. ^aAUC, area under the curve. ^bsensitivities, and specificities of each multimarker combination were compared with those of hCG + progesterone in the same column. ^cP < 0.05, ^dP < 0.01; ^eP < 0.05, hCG+progesterone+miR-873 compare to hCG+progesterone+miR-323 or hCG+progesterone+miR-873+miR-323

	AUC ^a (95% CI)	Sensitivity at:		Specificity at:	
		90% Specificity, %	95% Specificity, %	90% Sensitivity, %	95% Sensitivity, %
hCG	0.74 (0.65–0.84)	47.06	41.48	39.39	36.36
Progesterone	0.82 (0.73–0.91)	41.48	35.29	59.09	43.94
miR-218	0.39 (0.27–0.51)	17.65	5.88	31.82	3.03
miR-223	0.58 (0.47–0.69)	0	0	28.27	24.24
miR-141	0.38 (0.27–0.49)	11.76	0	34.85	33.33
miR-873	0.84 (0.76–0.92)	61.76	55.88	59.09	50.00
miR-323	0.74 (0.64–0.84)	47.06	32.35	43.94	34.85
hCG+progesterone ^b	0.84 (0.75–0.92)	55.88	44.12	59.09	48.48
hCG+progesterone+miR-323	0.87 (0.79–0.94)	67.65 ^c	50.00 ^c	63.64	56.06
hCG+progesterone+miR-873	0.91 (0.86–0.97)	79.41 ^{d,e}	64.71 ^{d,e}	84.85 ^{d,e}	80.82 ^{d,e}
hCG+progesterone+miR-873+miR-323	0.92 (0.87–0.98)	70.59 ^d	52.94 ^c	66.67	50.00

Fig. 3. Detection of single markers and multiple makers in EP and non-EP patients. (A) ROC curve analysis of hCG, progesterone, miR-218, miR-233, miR-141, miR-873, and miR-323 as single markers in the validation set of 34 patients with EP and 66 non-EP controls. (B) ROC curve analysis of hCG and progesterone; hCG, progesterone, and miR-873; hCG, progesterone, and miR-323; and hCG, progesterone, miR-873, and miR-323 as multiple markers in the validation set of 34 patients with EP and 66 non-EP controls, respectively.

44.12% for hCG plus progesterone at specificities of 90% and 95%, respectively ($P < 0.01$ and $P < 0.05$, respectively). Moreover, the combination of hCG, progesterone and miR-873 produced higher sensitivities than the combinations of hCG, progesterone, and miR-323 and the combinations of hCG, progesterone, miR-323, and miR-873 (both $P < 0.05$, at specificities of 90% and 95%, respectively).

Discussion

Approximately 15% of pregnancies are associated with complications such as preeclampsia, EP, gestational diabetes mellitus, spontaneous abortion, and preterm delivery among others [2, 16–18]. EP is an important cause of maternal morbidity and can be fatal when

undetected because of the potential for tubal rupture and hemorrhage[19]. The diagnosis of EP currently relies on a combination of sonographic findings and serum hCG measurements; however, EP is difficult to diagnose at an early stage because approximately one third of all cases have no clinical signs and 9% of patients have no symptoms prior to rupture[20]. In addition, single hCG measurements cannot distinguish between EP, SA, and VIP, requiring repeated hCG measurements that are not only time consuming and costly, but are also associated with a risk of rupture between measurements [19]. Therefore, the identification of accurate serum biomarkers for the early diagnosis of EP would be of great clinical value. However, research needs to be carried out in women, as there are no suitable animal models of tubal ectopic pregnancy, and serum concentrations of putative biomarkers are measured retrospectively in women who have been diagnosed with EP, VIP, and SA, which is associated with problems in the interpretation of biomarker utility[19]. Serum concentrations of these biomarkers vary with GA, and although some markers can distinguish between EP and VIP, they cannot discriminate between EP and SA [19]. Nevertheless, many markers have been tested for their capacity to detect EP at an early stage. Serum biomarkers of EP include those indicative of abnormal embryo/trophoblast growth (hCG, pregnancy-associated plasma protein-A, and activin-A among others) abnormal corpus luteum function (progesterone, estradiol), markers of growing pregnancy in the Fallopian tube, markers of inflammation and peritoneal irritation, and uterine markers of normal implantation, which would be disrupted in the presence of tubal implantation. Among these, hCG is the only biomarker routinely used in the clinic, and progesterone is sometimes added to the routine hCG/ultrasound diagnostic protocol despite controversies regarding its accuracy[19]. Activin A, which is not used in clinical practice, is a potentially important biomarker that is currently being investigated [21, 22].

miRNAs have shown potential as biomarkers for the detection of pregnancy-related diseases. The value of miRNAs lies not only in their noninvasive nature, but also in the fact that they are stable and their concentrations do not vary throughout pregnancy[16]. In 2008, Chim et al. compared the profiles of 157 miRNAs between third-trimester placental tissues and the corresponding maternal blood cells and identified 34 miRNAs that were present at more than ten-fold higher concentrations in maternal blood cells [23]. Of these, four miRNA (miRs-141, 149, 299-5p, and 135b) were confirmed to be of placental origin. Subsequent studies identified many placental-specific miRNAs, confirming that these molecules are released into the maternal circulation and could serve as serum biomarkers. To detect miRNAs with diagnostic potential for pregnancy-associated complications, researchers have compared the serum concentrations of miRNAs between patients with specific disorders and healthy controls, which resulted in the identification of a large number of differentially expressed miRNAs, many of which were confirmed as promising biomarkers for specific disorders. Zhao et al. examined the diagnostic accuracy of miRNAs for the detection of EP and showed that miR-517a, miR-519d, and miR-525-3p were significantly lower, whereas miR-323-3p was significantly higher in EP and SA than in VIP[8]. Furthermore, the combined assessment of miR-323-3p, hCG, and progesterone showed a sensitivity of 77.8 % for the detection of EP at a fixed specificity of 90%. In the present study, we measured the concentration of 21 pregnancy-associated miRNAs as well as hCG and progesterone in serum samples from patients with EP, VIP, and SA. Of five miRNAs differentially expressed between these patients, miR-873 and miR-223 were significantly downregulated in EP compared with SA and did not change significantly according to GA. MiR-323 was also significantly higher in EP than in VIP and SA, similar to a previously study[8], and the combined assessment of miR-323-3p, hCG, and progesterone showed a sensitivity of 67.65 % for the detection of EP at a fixed specificity of 90%, and in combination with other markers showed that the combination of miR-323 with hCG and progesterone and miR-873 had the highest AUC for the prediction of EP. As a single marker, miR-873 had the highest sensitivity at 61.76 % (at a fixed specificity of 90%), suggesting its potential as a biomarker for the early detection of EP. And combination with hCG, and progesterone showed a sensitivity of 79.41 % for the detection of EP at a fixed specificity of 90%. However, combination with hCG, progesterone, miR-873 and miR-323

showed a sensitivity of 70.59 % for the detection of EP at a fixed specificity of 90%.

miR-873 was previously shown to be among five miRNAs downregulated in EP in a miRNA microarray comparison of pregnancy derived tissues from patients with EP and those with voluntary termination of pregnancy (VTOP) [24], which is consistent with the results of the present study. However, Dominguez et al. showed that miR-223 was upregulated in EP, which differs from our results showing that miR-223 expression was significantly lower in EP than in SA. In the study by Dominguez et al. the upregulation of miR-223 was further validated by real-time PCR in a larger sample of embryonic tissues from EP and VTOP, and it was further shown to target GALNT1, a gene in the mucin biosynthesis pathway. Nevertheless, the study by Dominguez et al. did not compare EP and SA [24]; therefore, whether the results of the two studies are in accordance is difficult to determine. Zhi et al. showed that miR-223 was shown to inhibit bladder cancer cell migration and invasion by downregulating its target ZEB1 [25]. In addition, miR-223 was shown to be a potential target for the treatment of ischemic arrhythmias, as miR-223 repressed the expression of the voltage-gated K⁺ channel Kv4.2 [26]. miR-223 suppresses cardiomyocyte hypertrophy by modulating the expression of its target TNNT3K, a troponin interacting kinase [27]. In a study identifying differentially expressed miRNAs in polycystic ovarian syndrome, miR-223 was found to be downregulated and associated with increased risk of ovarian hyperstimulation syndrome [28]. Exosomes mediates miR-223 could inhibit Platelet-Derived Growth Factor Receptor-Beta (PDGFRβ) expression in Vascular Smooth Muscle cells [29]. miR-323 was shown to upregulate VEGF-A and increase vascularization in prostate cancer [30]. In a recent study, miR-873 was shown to be downregulated in breast tumors and to play a tumor suppressor role by targeting cyclin-dependent kinase 3, leading to the inhibition of ER-α activity and the suppression of breast cancer cell proliferation and tumor growth [31]. Insulin-like growth factor 2 mRNA binding protein 1 (IGFBP1) was identified as a target of miR-873 in glioblastoma [32]. IGFBP1, which is produced at high levels in the human endometrium, has been suggested as a marker of EP [33, 34]. IGF-I expression in the endometrium is induced by estrogen and mediates estrogen action, and IGF-II expression is associated with endometrial differentiation. IGFBP1 regulates the function of IGF at the embryo-endometrial interface. The modulation of IGFBP1 by miR-873 and their potential role in EP therefore merits further investigation.

In conclusion, we screened potential miRNA biomarkers for the detection of EP in an ED setting by analyzing a population of miRNAs with a known relationship to pregnancy or placental expression and by comparing their levels between patients with EP, VIP and SA. We identified five differentially expressed miRNAs and showed that miR-873 had the highest specificity as a single marker and is therefore a promising candidate biomarker for the early detection of EP. Further studies with a larger cohort are necessary to validate our results. Furthermore, assessment of other markers such as hCG and progesterone in combination with miRNAs may help improve the diagnostic performance of the identified miRNAs as biomarkers and should be performed in future studies.

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Disclosure Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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