Identification of candidate microRNA markers of endometriosis with the use of next-generation sequencing and quantitative real-time polymerase chain reaction

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Objective: To identify novel candidate diagnostic microRNA (miRNA) markers of endometriosis by means of an unbiased search with confirmation by means of targeted polymerase chain reaction (PCR).
Design: Retrospective cohort.
Setting: University teaching hospitals.
Patient(s): Women with endometriosis and control women, confirmed with the use of laparoscopy.
Interventions(s): Diagnostic laparoscopy and blood sample.
Main Outcome Measure(s): Next-generation sequencing (NGS) and quantitative real-time PCR (qRT-PCR).
Result(s): Candidate miRNAs differentially expressed in women with endometriosis compared with control women were identified by means of NGS and selected for qRT-PCR. Plasma samples from another cohort of women with surgically confirmed endometriosis (n = 53) and disease-free control women (n = 53) were checked for hemolysis using spectrophotometry and the ratio of miR-23a and miR-451 by means of qRT-PCR. MicroRNA signatures were quantified by means of qRT-PCR in hemolysis-free plasma samples of case subjects (n = 25) and control subjects (n = 28) with the use of miRcury LNA miRNA. Circulating levels of eight miRNAs (miR-199a-3p, miR-143-3p, miR-340-5p, let-7b-5p, miR-21-5p, miR-17-5p, miR-20a-5p, and miR-103a-3p) were significantly lower in case subjects compared to control subjects. The sensitivity and specificity for individual miRNAs ranged from 0.36 to 1.00 and from 0.43 to 1.00, respectively, but when combined produced sensitivity and specificity of 0.92 and 0.86 with positive (PPV) and (NPV) predictive values of 0.85 and 0.92, respectively. However, combination of five miRNAs (miR-17-5p, miR-20a-5p, miR-199a-3p, miR-143-3p, and let-7b-5p) produced sensitivity and specificity of 0.96 and 0.79 with PPV and NPV of 0.80 and 0.96, respectively.
Conclusion(s): We conclude that a panel of candidate miRNAs was comparable to laparoscopy in distinguishing between women with endometriosis and control women. (Fertil Steril® 2020;113:1232–41. ©2020 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: MicroRNA, miRNA, endometriosis, diagnosis, markers, plasma, blood

Endometriosis is a common chronic, benign, estrogen-dependent gynecologic disorder with prevalences of 1%–10% in women of reproductive age and 35%–50% in women with infertility and pelvic pain (1–3). Endometriosis is characterized by the growth of endometrial stromal cells and glands outside of the uterine cavity (1). The lack of a diagnostic blood test leads to diagnostic delays of an average 7 years (4, 5), with laparoscopy, either alone or in combination with histopathologic evidence of endometrial stroma and glands, remaining the criterion standard for the diagnosis of endometriosis (6). Laparoscopy is an expensive and invasiveness procedure whose risks, though rare, are serious. Consequently, diagnostic markers of endometriosis have been sought to provide more timely access to appropriate effective treatment alternatives for endometriosis.

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However, no clinical marker of endometriosis, either alone or in combination, has provided adequate sensitivity or specificity for the diagnosis of endometriosis (7–10). Thus, the search for suitable diagnostic markers of endometriosis remains a high but unmet research priority (11).

Recently, several groups (12–19) explored the potential of circulating microRNA (miRNA) levels for the diagnosis of endometriosis. miRNAs are single-stranded RNA molecules 21–25 nucleotides in length that act as posttranscriptional silencers of gene expression by degradation of their target RNAs (20). MicroRNAs are present in body fluids, including blood, either contained in exosomes or bound to protein complexes, which makes them more stable than circulating hormone or cytokine concentrations and therefore better candidate markers of disease (21). One miRNA can target several genes, and one gene can be targeted by different miRNAs (22). Circulating levels of miRNAs are dysregulated in several different cancers (23–25) and in endometriosis (12–19). Although circulating levels of multiple miRNAs differ significantly between women with endometriosis and control women, the results have largely not been reproduced by other investigators, and so miRNAs may have limited potential as diagnostic markers of endometriosis (19). However, we suggest that the lack of reproducibility is likely the consequence of different methods, including in situ hybridization, targeted real-time polymerase chain reaction (RT-PCR), and different screening platforms, including miRNA-based microarrays, next-generation sequencing (NGS), and bioinformatics followed by RT-PCR validation (12–19, 26–32). Moreover, we postulate that different reference materials used to quantify circulating miRNA levels are an additional source of variation. Although the small nuclear RNA RNU6 has been widely used in the general miRNA literature to normalize miRNA expression in tissue, abundance and stability of expression have not been evaluated for circulating miRNA expression in women with endometriosis. Furthermore, RNU6 has low stability and abundance that is greatly influenced by sample storage and processing, and the crossing point (Cp) values of RNU6 are highly variable from miRNA Cp values (19, 33, 34). Similarly, the abundance and stability of miR-16-5p levels in the serum of women with endometriosis is uncertain but variable from the Cp values of miRNA targets (19). Furthermore, circulating levels of miR-16-5p are altered by inflammation and stress (35, 36). Therefore we suggest that neither RNU6 nor miR-16-5p is suitable for normalization of circulating miRNA levels in women with endometriosis. Thus the potential of miRNA in the diagnosis of endometriosis remains unresolved.

NGS offers the advantage of screening samples for known as well as novel miRNAs in an unbiased manner (13, 14, 23, 37, 38). Recently, NGS was used to screen for miRNAs that are differentially expressed in the plasma (30) and extracellular vesicles (39) of women with endometriosis compared to control women. We recently used miRseq NGS in a preliminary study of plasma samples collected during the early secretory phase as follows: 1) women (n = 5) with stage I–II endometriosis; 2) women (n = 5) with Stage III–IV endometriosis; 3) women without endometriosis (control; n = 5); and 4) women without endometriosis but other endometrial pathologies (e.g., adenomyosis, myomas, and leiomyomas; n = 5). From that preliminary study, plasma levels of 28 novel miRNAs and 41 miRNAs differentially expressed in women with endometriosis compared with the control group were identified. Results from NGS were validated by means of quantitative (q) PCR in 45 women with surgically confirmed endometriosis compared with control women. Consequently, the objective of the present study was to expand our assessment of miRNAs identified in our preliminary screen for candidate markers of endometriosis. Herein, we describe the results of qRT-PCR analysis for candidate miRNAs as clinical markers of endometriosis in a novel cohort of women surgically diagnosed with endometriosis compared with control women.

MATERIALS AND METHODS

Choosing the Candidate List of miRNAs to Be Evaluated with qRT-PCR

A preliminary NGS (NextSeq50, v2 sequencing) study was conducted in plasma samples collected in the early secretory phase of the menstrual cycle from women with surgically confirmed endometriosis (n = 10) and a control group of women without endometriosis (n = 10). The NGS results were validated by means of qPCR in a training set of plasma samples from women with surgically confirmed endometriosis (n = 30) and a control group of women without endometriosis (n = 15). In the present study, 20 miRNAs identified in our previous NGS study with plasma levels that differed either significantly ($P \leq .01$) or by at least twofold change between women with endometriosis and control women were included in our analysis. In addition, four miRNAs identified in ongoing studies in our laboratory were selected for qRT-PCR to yield a panel of 24 candidate miRNAs for this study. Thus, candidate miRNAs in the current study consisted of 20 miRNAs identified in our preliminary study and several miRNAs identified through our ongoing miRNA studies in endometriosis. Reference miRNAs with stable and equivalent plasma levels in women with endometriosis and control women were used to standardize circulating plasma miRNA results, in which confounding from hemolysis was excluded.

Study Population

The study protocol was reviewed and approved by the Human Research Ethics Committee on Biomedical Research of the University of Tarbiat Modares (Tehran, Iran). All study participants provided written informed consent before entering the study. To determine the appropriate sample size to detect a significant difference in plasma miRNA levels between women with surgically confirmed endometriosis and control women, we anticipated that we could expect a difference of approximately twofold in mean plasma miRNA level based on results of previous related miRNA studies (12–19). A sample size calculation using a two-tailed *t* test to achieve 80% power, alpha of 5%, and SD of 0.56 revealed that we would need a minimum of ten study participants per group. Women 23–45 years of age who were undergoing diagnostic laparoscopy for indications including pelvic pain, infertility, pelvic mass, and uterine leiomyomas, without symptoms or history of inflammatory, systemic, or chronic disease, were invited to participate in this study. Laparoscopies were carried out by minimally invasive surgeons with extensive experience in the diagnosis and treatment of endometriosis.

Diagnosis of endometriosis was confirmed with the use of histopathology. All participants reported that they were not active smokers and had not received hormonal medications during the past 3 months before diagnostic laparoscopy. Furthermore, none of the subjects reported using nonsteroidal antiinflammatory drugs or other antiinflammatory medications before surgery. Because we and others have found that circulating miRNA levels are not influenced by menstrual cycle stage (12, 14, 16, 17, 19), study participants from all menstrual cycle phases were included in this study.

Blood Sample Collection and Processing

Blood samples were collected at Arash Women's Hospital, Tehran. Briefly, a 5-mL peripheral blood sample was collected in K3-EDTA plasma separator tubes from 106 candidates after induction of anesthesia for laparoscopy. Blood samples were immediately transferred to the laboratory and centrifuged at 1,500*g* for 10 minutes at room temperature. The supernate was removed and recentrifuged at 1,500*g* for 10 minutes at room temperature. Plasma was transferred to 1.8-mL cryovials and stored at −80°C until required for analysis. Plasma samples from study participants with surgical diagnosis of endometriosis confirmed by pathology were assigned to the case group (n = 53) and samples from women without surgical evidence of endometriosis were assigned to the control group (n = 53).

Hemolysis Check

Plasma samples were examined for hemolysis based on a two-step method. First, absorbance was measured at 414 nm with the use of a spectrophotometer (Thermo Scientific Nanodrop) and samples with results ≤0.2 were selected for RNA extraction. To identify samples with hemolysis, plasma samples were further tested for expression levels of two miRNAs: miR-451 and miR-23a. MiR-23a is known to have stable expression levels in plasma, whereas miR-451 is known to be expressed at high levels in red blood cells. After RNA extraction and cDNA synthesis, the delta quantification cycle (Cq) values for miR-23a-451 were calculated for each sample. Samples with delta Cq <7 for these two miRNAs were selected as hemolysis-free samples for further analyses.

Spectrophotometry results revealed that 104 of the 106 plasma samples (98.1%) had absorbance results ≤0.2, suggesting absence of hemolysis. The delta Cq ratios for miR-23a and miR-451 were ≤7.0 in 40 of the 52 women with endometriosis (76.9%) and in 36 of the 52 control women (69.2%) and thus hemolysis free. Based on better results, 25 women with endometriosis and 28 control women were selected for further analysis for a combined total of 53 of 104 plasma samples (51.0%) with absorbance ≤0.2 and delta Cq ≤7 and therefore considered to be free of hemolysis.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 200 μL plasma with the use of miRNeasy Serum/Plasma Advanced Kit (Qiagen) according to the manufacturer's instructions. For extraction quality control, we added 3.5 μL cel-miR-39 (Qiagen) diluted in 10 ng MS2 containing water to each extraction reaction according to the manufacturer's guide. RNA was eluted in 20 μL nuclease-free water. Extraction quality was monitored by adding cel-miR-39 during extraction, and after performing qPCR we found that SDs for cel-miR-39 Cq values were not >1.0 (SD 0.9) and there was no significant difference between case and control groups (P=.99). cDNA was made for each sample using miRcury LNA RT Kit based on the Poly(A) reverse-transcription PCR method. Four μL of elute (20 ng RNA) was used according to the manuals for 20 μL reverse-transcription PCR. cDNA synthesis quality was checked with the use of UniSP6 target, which was included in cDNA synthesis kit, and the subsequent quantification of this target by relevant primers on the plates. Standard deviation for related Cq values was SD 0.42, and no significant differences between case and control groups were found (P=.587).

MicroRNA Profiling with the Use of qRT-PCR

MiRcury LNA miRNA custom PCR panels were designed with the use of Qiagen online software, containing specific forward and reverse primers for the 24 candidate miRNAs and three candidate internal reference miRNA (miR-148b-3p, miR-30e-5p, and miR-103a-3p) plus cel-miR-39 (extraction control), and UniSP6 and UniSP3 (template control) were included on the panel (Table 1). Each primer was analyzed in duplicate. cDNA was diluted 10× with the use of nuclease free water, and a mixture of 5 μL miRcury LNA Sybr Green, 4 μL diluted cDNA, and 1 μL nuclease-free water per reaction was prepared. qPCR was performed with the use of a Roche Light Cycler G 480, according to the miRcury LNA miRNA Custom PCR Panels handbook and the given qPCR program. Calculated mean Cq values and melt curves for each target were obtained from instrument software, and those Cq values were used for further analysis. We also included one negative template control (NTC) sample on the last plate. Cq values <35 were considered for data analysis. Also, the NTC was negative (no Cq values) for all primers. Data analysis was performed after calibration of each plate to eliminate all possible qPCR efficiency differences between plates using interplate calibrator (IPC) UniSP3 Cq values (SD 0.2) and calculation of calibration factor by means of the formula IPC plate − IPC overall and then subtracting the calibration factor from all Cq values in each plate. Cq values for each miRNA was normalized to average Cq values of two reference miRNAs (hsa-miR-148b-3p and hsa-miR-30e-5p). The $2^{\Delta Cq}$ ($2^{\text{Cq of reference miRNAs – Cq of miRNA of interest}}$) value was calculated for each miRNA in each sample.

Selection of Reference Genes

The mean Cq values for miRNAs hsa-miR-148b-3p and hsa-miR-30e-5p showed no significant differences between women with endometriosis and the control group (P=.894). The mean value for the combination of hsa-miR-148b-3p, hsa-miR-30e-5p, and hsa-miR-103a-3p was P=.838, but the plasma levels of one candidate reference miRNA (hsa-miR-103a-3p) was significantly different between the two groups (P=.007) when it was compared with the average Cq values of the three candidate reference genes (including its own) or when it was compared with the mean Cq values of the other two candidate reference gene. Consequently, hsa-

TABLE 1

List of candidate miRNA biomarkers and controls on the custom designed panel.

miRNA	#	miRNA ID	Assay catalog no.
miRNA markers of endometriosis evaluated in this study	1	hsa-miR-150-5p	YP00204660
	2	hsa-miR-199a-3p	YP00204536
	3	hsa-miR-143-3p	YP00205992
	4	hsa-miR-199a-5p	YP00204494
	5	hsa-miR-335-3p	YP00205613
	6	hsa-miR-381-3p	YP00205887
	7	hsa-miR-224-5p	YP00204641
	8	hsa-miR-340-5p	YP00206068
	9	hsa-let-7d-3p	YP00205627
	10	hsa-miR-92a-3p	YP00204258
	11	hsa-miR-221-3p	YP00204532
	12	hsa-miR-486-5p	YP00204001
	13	hsa-let-7b-5p	YP00204750
	14	hsa-miR-122-5p	YP00205664
	15	hsa-miR-21-5p	YP00204230
	16	hsa-miR-133a-3p	YP00204788
	17	hsa-miR-148a-5p	YP00204188
	18	hsa-let-7a-3p	YP00206084
	19	put-miR-5	YCP0043333
	20	put-miR-27	YCP0043336
	21	hsa-miR-125b-5p	YP00205713
	22	hsa-miR-17-5p	YP00204771
	23	hsa-miR-20a-5p	YP00204292
	24	hsa-miR-3613-5p	YP02119046
	25	hsa-miR-103a-3p[a]	YP00204063
Reference miRNAs	26	hsa-miR-30e-5p	YP00204714
	27	hsa-miR-148b-3p	YP00204047
Extraction control	28	cel-miR-39–3p	YP00203952
Template control	29	unisp3	YP02119288
	30	unisp6	YP00203954

[a] Hsa-miR-103a-3p was initially investigated as a candidate reference miRNA but preliminary results revealed that it was differentially expressed in women with endometriosis compared with the control group and therefore was included as a candidate marker of endometriosis.

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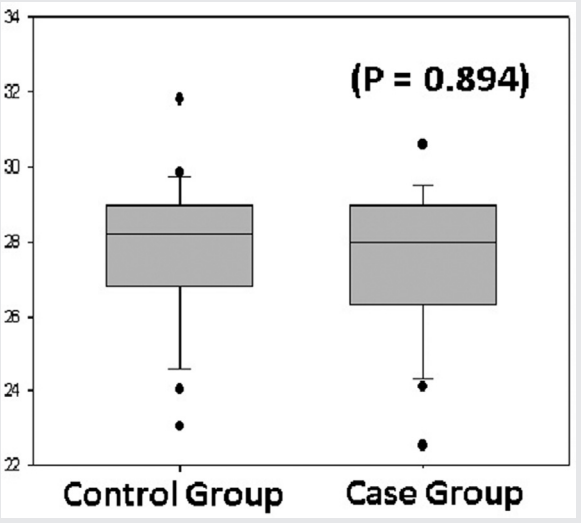
FIGURE 1



Box plots of the median quantification cycle values with the 25th and 75th percentiles and 95% confidence intervals for the two miRNAs (hsa-miR-148b-3p and hsa-miR-30e-5p) used as references for standardizing results between plasma miRNA levels in women with endometriosis (n = 25) and a control population (n = 28). Results were compared by means of Mann-Whitney *U* test and revealed that plasma levels of the selected miRNAs were equivalent (*P*=.894) in both study groups and therefore suitable for use as reference markers.

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miR-103a-3p was excluded as a reference gene but included in all subsequent comparisons as a candidate marker of endometriosis. Furthermore, the mean Cq values of the two reference miRNAs (hsa-miR-148b-3p and hsa-miR-30e-5p) were used as reference miRNAs (Fig. 1) to standardize miRNA results in women with endometriosis compared with the control group.

Statistical Analysis

Data are presented as mean ± SD for age of study participants and compared by *t* test. Chi-square analyses was performed for comparison of proportions of study subjects reporting dysmenorrhea, subfertility, and familial history of infertility between the two groups with the use of SPSS 23. The relative miRNA levels were obtained with the use of $2^{\Delta Cq}$ ($2^{Cq\ of\ reference\ miRNAs\ -\ Cq\ of\ miRNA\ of\ interest}$) for each miRNA. Delta Cq values for women with endometriosis and controls were compared for each miRNA by means of Mann-Whitney *U* test with the use of Sigmaplot v14.0 (SSI). Each miRNA with a *P* value $<.05$ was considered to be statistically significant. For differentially expressed miRNAs between case and control groups, receiver operating characteristic (ROC) curves were prepared and the areas under the curve (AUCs) calculated with the use of NCSS software (2019). To find the best combination of miRNAs that discriminated between women with and without endometriosis, logistic regression was performed in SPSS 23 for different miRNA combinations. ROC curves were depicted for each combination of miRNAs and the best sensitivity (true positive rate), specificity (true negative rate), positive predictive value (PPV), and negative predictive value (NPV) were identified at the threshold indicated by the highest calculated Youden index.

RESULTS

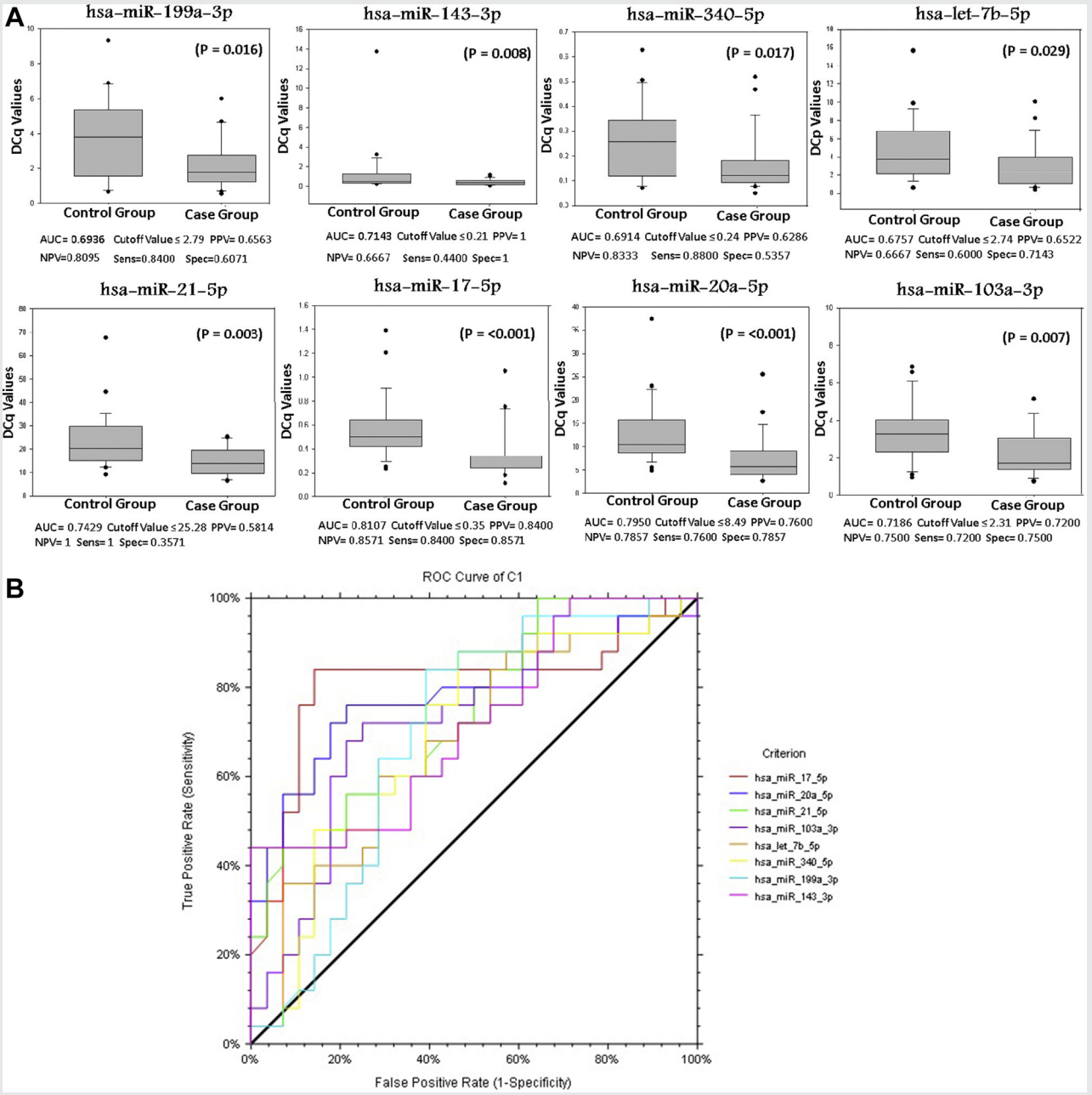
Study Participant Characteristics

The mean age of women with endometriosis (35.7 ± 4.5 y) did not differ significantly (*P*=.297) from the control group (34.0 ± 6.5). In women with endometriosis (n = 25), the majority were diagnosed with revised American Fertility Society stage III–IV and three women had endometriomas. In the control group, evaluation of the uterus revealed normal findings (n = 2), abnormal uterine morphology (n = 9), presence of myomas (n = 6), fibroma (n = 1), cysts (6), and obstructed fallopian tubes but no evidence of endometriosis. Dysmenorrhea was significantly more common (*P*=.006) among women with endometriosis (11 of 25, 44%) compared with the control group (3 of 28, 10.7%). The percentages of women who reported infertility (*P*=.61), subfertility (*P*=.06), and family history of infertility (*P*=.486) were not significantly different between the two study groups.

Circulating miRNA Levels in the Case and Control Groups

One of the two candidate miRNA sequences, which was found in our previous NGS study, was expressed in all samples of the present study, showing Cq values ranging from 24.73 to 35 (SD 2.48). Plasma levels of eight miRNAs of the 24 tested miRNAs were significantly different in women with endometriosis compared with control women. miR-199a-3p, miR-143-3p, miR-340-5p, let-7b-5p, miR-21-5p, miR-17-5p, miR-20a-5p, and miR-103a-3p were significantly lower in women with endometriosis compared with control women (Fig. 2A). The sensitivity and specificity for individual miRNAs ranged

FIGURE 2



(**A**) Box plots of the relative plasma levels for each of the eight miRNAs (hsa-miR-199–3a, hsa-miR-143-3p, hsa-miR-340-5p, hsa-let-7b-5p, hsa-miR-21-5p, hsa-miR-17-5p, hsa-miR-20a-5p, and hsa-miR-103a-3p) that were significantly lower in women with surgically confirmed endometriosis (n = 25) compared with the control population (n = 28). Data presented represent the median delta quantification cycle (DCq) values with the 25th and 75th percentiles and 95% confidence intervals. Results were compared by means of Mann-Whitney *U* test, and the corresponding area under the receiver operating characteristic curve (AUC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each individual marker are shown beneath the graph. (**B**) Receiver operating characteristic curves for each of the eight miRNAs with plasma levels that were significantly lower in women with endometriosis compared with the control population.

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from 0.36 to 1.00 and from 0.43 to 1.00, respectively. To define the diagnostic potential of each of the eight miRNAs with significantly different plasma levels, ROC curves were generated (Fig. 2B).

Logistic regression of delta Cq values followed by ROC curve analysis for the eight miRNAs with plasma levels that differed between case and control subjects produced the AUC, optimal Youden index, cutoff, sensitivity, specificity, PPV, and NPV for each combination. We found that the combination of all eight miRNAs provided an AUC of 0.9486 with sensitivity and specificity of 0.92 and 0.86 and PPV and NPV of 0.85 and 0.92, respectively (Fig. 3A). However, the combination of five miRNAs (miR-17-5p, miR-20a-5p, miR-199a-3p, miR-143-3p, and let-7b-5p) resulted in an AUC of 0.9286 with sensitivity and specificity of 0.96 and 0.79 and PPV and NPV values of 0.80 and 0.96, respectively (Fig. 3B).

DISCUSSION

Results of this study revealed a group of eight candidate miRNAs that were significantly lower in the plasma samples of women with endometriosis compared with control women. However, the combination of five miRNAs (miR-17-5p, miR-20a-5p, miR-199a-3p, miR-143-3p, and let-7b-5p) produced an epigenetic signature with sensitivity and specificity (0.96 and 0.79, respectively) equivalent to laparoscopy (10) in discrimination between women with endometriosis and control women. We also found a group of three candidate reference miRNA with levels that were stable across all women with endometriosis and controls, and that were also not influenced by the course of the menstrual cycle. By further checking expression differences between two groups for each of these candidate reference miRNAs, we excluded hsa-miR-103a-3p because we found that it was differentially expressed in women with endometriosis compared with the control group of women without endometriosis. Therefore, we chose two miRNAs (hsa-miR-148b-3p and hsa-miR-30e-5p) to be used as internal reference miRNAs.

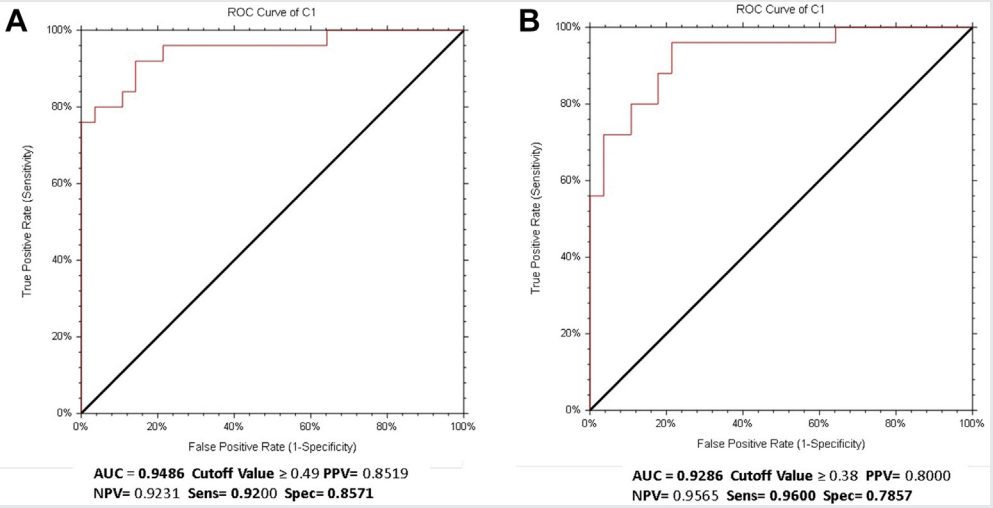
In the present study, we used NGS, an unbiased sequencing technique, followed by qRT-PCR to identify novel miRNA markers of endometriosis, an approach that diverged from the majority of previous studies in which commercially available miRNA microarrays were used but that was consistent with recent studies (19, 26, 30). We found that plasma levels of three miRNAs (hsa-miR-21-5p, hsa-miR-143-3p, and hsa-miR-199a-3p) were significantly lower in women with endometriosis compared with the control group; however, circulating levels were higher in a NGS survey that we had conducted previously. In our previous NGS study, analyses were performed on pooled plasma samples from women with endometriosis and a control group in the early secretory phase of the menstrual cycle. In the present study, we used individual plasma samples regardless of menstrual cycle phase. Furthermore, similarly to a recent study (30), we used stable reference miRNA instead of reference materials (RNU6 and miR-16) whose value has been challenged (34, 36). In the present study, care was taken to identify reference miRNAs that were abundantly expressed in the plasma with levels that were equivalent in women with endometriosis and control women. Moreover, we chose hemolysis-free samples for analysis based on spectrometry results ≤ 0.2 and delta Cq ratios of miR-23a and miR-451a ≤ 7.0 to mitigate potential confounding that could arise from sample hemolysis (40, 41). Indeed, circulating levels of several miRNAs have been shown to be 13 times higher in samples with hemolysis (41). We propose that the use of hemolysis-free samples and validated reference miRNAs to standardize miRNA results are important steps to improve reproducibility.

In the present study, plasma levels of eight miRNAs (miR-17-5p, miR-20a-5p, let-7b-5p, miR-21-5p, miR-199a-3p, miR-143-3p, miR-103a-3p, and miR-340-5p) were significantly lower in women with endometriosis compared with the control group. The finding that circulating levels of miR-17-5p, miR-20a-5p and let-7b-5p were significantly lower in women with endometriosis compared with the control group are harmonious with previous studies (13, 15). In contrast, the finding that plasma levels of miR-199a-3p and miR-143-3p were significantly lower in women with endometriosis compared with the control group conflict with previous studies (12, 14). Both of those studies used RNU6 as a reference to standardize results for comparison, but RNU6 levels have previously been reported to be unstable, unreliable, and a poor reference for circulating miRNA levels because it is not processed or protected in the same way as miRNAs (34, 36). Therefore, we suggest that divergent results are most likely the result of using different reference materials to standardize study results. We therefore further propose that in addition to controlling for hemolysis that the use of reference miRNAs with levels in the circulation that are both abundant and unaffected by disease status are likely to yield reproducible results that will enhance the as yet unrealized diagnostic utility of miRNAs.

We found that the sensitivity and specificity for the individual miRNAs with circulating levels that differed significantly between women with endometriosis and control women in the present study were low and consistent with previous studies. Specifically, circulating miR-17-5p were lower in women with endometriosis, with sensitivity and specificity of 60% and 90%, respectively (15). Similarly, plasma levels of miR-20a-5p were lower in women with endometriosis compared to the control group with sensitivity and specificity 60% and 90%, respectively (15). However, we found that the sensitivity and specificity of the markers were improved when combined in a panel, results that are consistent with a previous study (12). In the present study, a panel of eight miRNA markers of endometriosis distinguished between women with endometriosis and disease-free control women with sensitivity and specificity of 0.92 and 0.86 and PPV and NPV of 0.85 and 0.92, respectively. However, the combination of five miRNAs (miR-17-5p, miR-20a-5p, miR-199a-3p, miR-143-3p, and let-7b-5p) produced sensitivity and specificity of 0.96 and 0.79 with PPV and NPV values of 0.80 and 0.96, respectively. These findings are similar to the sensitivity and specificity reported for laparoscopy (10) but are lower than those reported in another study (12) for a different panel of miRNA markers.

We suggest that the value of the miRNAs identified as candidate markers of endometriosis is enhanced by evidence

FIGURE 3



(**A**) Receiver operating characteristic (ROC) curve for the combination of all eight miRNAs with plasma levels that were significantly lower in women with endometriosis compared with the control population. The sensitivity and specificity of 0.95 and 0.86 with PPV and NPV of 0.85 and 0.92, respectively, are marginally lower than those of laparoscopy. (**B**) ROC curve for the five miRNA markers (has-miR-17-5p, has-miR-20a-5p, has-miR-199a-3p, has-miR-143-3p, and has-let-7b-5p) with plasma levels that were significantly lower in women with endometriosis compared with the control population and produced sensitivity and specificity of 0.96 and 0.79 with PPV and NPV of 0.80 and 0.96, respectively, which are equivalent to those of laparoscopy.

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of their involvement in regulating pathways thought to be important in the pathophysiology of endometriosis (46) and thus uncovering novel potential therapeutic targets. Plasma levels of both miR-17-5p and miR-20a-5p were lower in women with endometriosis compared with control women in the present study and play critical roles in the pathogenesis of endometriosis and its progression. Specifically, these two miRNAs are down-regulated in endometriotic tissue and plasma samples that causes their target genes *HIF1A*, *VEGFA*, *BCL2*, *CDKN1A*/p21, *CCND1* (cyclin D1), and *E2F3*, interleukin (IL) 8, and transforming growth factor (TGF) β to be expressed in higher levels, leading to hypoxia responses and inflammation, angiogenesis, cell proliferation and survival, lesion progression, and epithelial-mesenchymal transition (23, 47–55). Plasma levels of let-7b-5p were also lower in women with endometriosis compared with control women in the present study, and down-regulation of this miRNA in sera of patients compared with control subjects is associated with elevated levels of tumor necrosis factor (TNF) α and inflammation. Higher levels of TNF-α, IL-1β, IL-6, and IL-8 inflammatory cytokines were found in macrophage cultures transfected with let-7b-5p inhibitors (56). Down-regulation of let-7b in endothelium causes higher levels of TGF-β and its receptors, which enhances endothelium-mesenchymal transition (57). The let-7 family, including let-7b-5p, is down-regulated in the circulation as well as human endometrial stromal cells (HESCs), which induces higher levels of aromatase and K-Ras, and therefore we suggest that it is important in the pathophysiology of endometriosis (58). Introducing let-7b-5p locally to a mouse model down-regulated genes important in disease pathogenesis, such as *KRAS4A*, *KRAS4B*, *Cyp19a*, *ERα*, *ERβ*, and *IL6* (59).

The plasma levels of several miRNAs (miR-143-3p, miR-340-5p, miR-21-5p, and miR-103a-3p) were lower in women with endometriosis compared with control women in the present study but have no known function in the endometrium or endometriosis. MiR-143-3p is one of the most abundant miRNAs in both endometriotic and adjacent healthy tissues (60); however, miR-143-3p has no established role in the endometrium and endometriosis. In esophageal squamous cell carcinoma, miR-143-3p targets the *QKI5* gene, increasing proliferation, invasion, and epithelial-mesenchymal transition (61), all of which are important in the pathophysiology of endometriosis. Although the roles of miR-17-5p and miR-20a-5p in the pathophysiology of endometriosis remain to be established, these miRNAs have been implicated in the pathobiology of several cancers. MiR-21-5p was up-regulated in pancreatic and gastric cancers, hepatitis B virus–related hepatocellular carcinoma, and esophagitis (42–45). MiR-340-5p was reported to be up-regulated in recurrent breast cancer serum samples compared with a control group (25). MiR-340-5p, which is down-regulated in breast cancer cell lines, regulates c-Met, inducing higher levels of matrix metalloproteinase (MMP) 2 and MMP-9 and promoting invasion and migration (62). Further support for a role of miR-340-5p in endometriosis is provided by its down-regulation in melanoma cells under hypoxia conditions which in turn induces higher levels of *HIF2α*, *ABCB5*, *OCT4*, *SOX2*, and *ZEB1* products (63). MiR-103a-3p was initially investigated as a potential reference miRNA in the present study but we found that circulating levels were significantly lower in women with endometriosis compared with the control women. Therefore, we suggest that miR-103a-3p is a novel candidate marker of endometriosis. MiR-103a-3p

expression was down-regulated in gastric cancer cells and associated with cell proliferation, migration, and invasion by means of inhibition of c-Myb (64). Taken together, our results suggest that miR-103a-3p is important in the pathophysiology of endometriosis. However, although we found lower circulating levels of miR-103a-3p in the plasma of women with endometriosis compared with control women, the levels of this miRNA in plasma are sensitive to hemolysis (41) further highlighting the importance of controlling for potential confounding of results by hemolysis. Similarly to the previous miRNAs discussed, we found lower circulating levels of miR-21-5p in women with endometriosis compared with the control group, whereas, unlike the miRNAs previously discussed in this report, it is up-regulated in eutopic endometrium from women with endometriosis compared with control women and is linked with decreased apoptosis, with lower expression associated with higher apoptosis in HESCs (65). Moreover, miR-21-5p levels were higher in exosomes from endometriotic cells compared with control samples from secretory-phase endometrium; however, there was no significant difference in its expression intracellularly between these cells (66).

In summary, using an unbiased approach, we identified several candidate miRNA markers of endometriosis. While individually they did not have suitable sensitivity and specificity for the diagnosis of endometriosis, the combination of five miRNA markers had sensitivity and specificity similar to that of laparoscopy. Therefore, we suggest that our panel of five miRNAs are suitable candidate markers for validation as diagnostic tools in endometriosis.

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Identificación de candidatos microRNA como marcadores de endometriosis con el uso de secuenciación de próxima generación (NGS) y reacción en cadena de la polimerasa (PCR) cuantitativa en tiempo real

Objetivo: Identificar nuevos marcadores de diagnóstico de endometriosis micro ARN (miARN) mediante una búsqueda imparcial confirmada mediante reacción en cadena de la polimerasa dirigida (PCR)

Diseño: Estudio retrospectivo de cohortes.

Paciente (s): Mujeres con endometriosis y mujeres controles, confirmado con el uso de laparoscopia.

Intervenciones: laparoscopia diagnóstica y muestra de sangre.

Principales medidas de resultado: secuenciación de próxima generación (NGS) y PCR cuantitativa en tiempo real (qRT-PCR).

Resultado (s): Los candidatos de miARN expresados diferencialmente en mujeres con endometriosis comparadas con las mujeres control fueron identificados por medio de NGS y seleccionados para qRT-PCR. Muestras plasmáticas de otra cohorte de mujeres con endometriosis confirmada quirúrgicamente (n = 53) y de mujeres control libres de enfermedad (n = 53) fueron evaluadas para hemólisis utilizando espectrofotometría y la proporción de miR-23a y miR-451 mediante qRT-PCR. Las firmas de microARN se cuantificaron mediante qRT-PCR en muestras de plasma sin hemólisis de las sujetos caso (n = 25) y sujetos control (n = 28) con el uso de miRcury LNA miRNA. Los niveles circulantes de ocho miARN (miR-199a-3p, miR-143-3p, miR-340-5p, let-7b-5p, miR-21-5p, miR-17-5p, miR-20a-5p y miR-103a-3p) fueron significativamente inferiores en los casos en comparación con los controles. La sensibilidad y especificidad para miRNAs individuales osciló entre 0.36 y 1.00 y entre 0.43 y 1.00 respectivamente pero, de manera combinada, resultaron en sensibilidad y especificidad de 0.92 y 0.86 con valores predictivo positivo (PPV) y negativo (NPV) de 0,85 y 0,92 respectivamente. Sin embargo, la combinación de cinco miRNAs (miR-17-5p, miR-20a-5p, miR-199a- 3p, miR-143-3p y let-7b-5p) se tradujo en sensibilidad y especificidad de 0.96 y 0.79 con PPV y NPV de 0.80 y 0.96 respectivamente.

Conclusión (es): Se concluye que un panel de miRNAs candidatos fue comparable a la laparoscopia para discriminar entre mujeres con endometriosis y mujeres control.