Serum miR-451a Levels Are Significantly Elevated in Women With Endometriosis and Recapitulated in Baboons (*Papio anubis*) With Experimentally-Induced Disease

Reproductive Sciences
2017, Vol. 24(8) 1195-1202
© The Author(s) 2016
Reprints and permission:
sagepub.com/journalsPermissions.nav
DOI: 10.1177/1933719116681519
journals.sagepub.com/home/rsx

Warren B. Nothnick, PhD, HCLD^{1,2}, Tommaso Falcone, MD³,
Niraj Joshi, PhD⁴, Asgerally T. Fazleabas, PhD⁴, and Amanda Graham, BS¹

Abstract

We have previously demonstrated that human microRNA-451a (miR-451a) endometriotic lesion expression is significantly higher compared to that of the corresponding eutopic endometrium. The objective of the current study was to examine the relationship between lesion and serum content of miR-451a and to determine the utility of serum miR-451a in distinguishing between women with and without visible signs of endometriosis. Eighty-one participants were enrolled in this study, 41 with confirmed endometriosis and 40 without visible signs of endometriosis at laparoscopy (n = 20) or symptoms of endometriosis (pain, infertility n = 20). Experimental endometriosis was also induced in 8 baboons. Blood, endometriotic lesions, and eutopic endometrial samples were collected from women undergoing laparoscopy for surgical removal of endometriosis. Blood was also collected from control participants with no signs and symptoms associated with the disease as well as from baboons prior to, and then 1, 3, 6, 9, and 15 months postinduction of endometriosis. MicroRNA-451a was assessed by quantitative real-time polymerase chain reaction in all samples. In humans, serum miR-451a levels positively correlated with endometriotic lesion miR-451a content, and sera levels were significantly higher in these participants compared to controls. The area under the curve (AUC) for miR-451a was 0.8599. In baboons, serum miR-451a reached statistically significant peak levels at 6 months postinduction of endometriosis. We conclude from this study that sera miR-451a levels positively correlated with endometriotic lesion content and are significantly greater compared to sera levels in women without visible signs or symptoms of endometriosis. MicroRNA-451a may serve as a serum diagnostic marker for endometriosis.

Keywords

endometriosis, miRNA, miR-451a, serum, endometriotic lesion

Introduction

Endometriosis is a debilitating disease which affects as many as 10% of reproductive-age women and is characterized by primary complaints of pelvic pain, dysmenorrhea, and infertility.¹ Defined as the presence of ectopic endometrial stromal and glandular tissue, endometriosis is thought to develop via reverse menstruation of viable endometrial tissue into the peritoneal cavity. However, because almost all reproductive-age women exhibit some degree of retrograde menstruation,¹⁻³ it is postulated that additional, yet unidentified, factors must contribute to the development and progression of the disease.

MicroRNAs (miRNAs) have been proposed to play a role in the pathogenesis of endometriosis, but beyond initial characterization, our understanding on their role in this disease is just beginning to evolve. Defined as a class of small noncoding regulatory RNAs that regulate gene expression posttranscriptionally,^{4,5} miRNAs have been implicated to play a vital role in cellular events, many of which are conducive to endometriosis

development, including cell proliferation, invasion, and apoptosis.⁶ MicroRNA expression profiles have been established for endometriosis in both the disease tissue and eutopic endometrium as well as control patients.⁷⁻¹¹

¹ Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS, USA

² Center for Reproductive Sciences, Institute for Reproductive Health and Regenerative Medicine, University of Kansas Medical Center, Kansas City, KS, USA

³ Department of Obstetrics, Gynecology and Women's Health Institute, Cleveland Clinic, Cleveland, OH, USA

⁴ Department of Obstetrics and Gynecology and Reproductive Biology, Michigan State University, Grand Rapids, MI, USA

Corresponding Author:

Warren B. Nothnick, Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Blvd, HLSIC 3095, Kansas City, KS 66160, USA.

Email: wnothnic@kumc.edu

We recently reported that microRNA-451a (miR-451a; formerly miR-451) is overexpressed in endometriotic lesion tissue compared to matched eutopic endometrium.¹² Several factors, which are misexpressed in endometriosis and proposed to play a role in the pathogenesis of endometriosis, have been validated as targets of miR-451a. These factors include macrophage migration inhibitory factor (MIF),¹²⁻¹⁴ 14-3-3 protein zeta/delta (YWHAZ),¹⁵ ras-related protein 14 (RAB14),^{16,17} calcium binding protein 39 (CAB39),¹⁸ cyclin-dependent kinase inhibitor 2D (CDKN2D),¹⁹ mitogen-activated protein kinase 1 (MAP3K1),¹⁹ and proteasome subunit beta type-8/20S proteasome subunit beta-5i (PSMB8),²⁰⁻²² all of which have been shown to be associated with cellular proliferation and survival, with MIF and YWHAZ being examined in endometriotic tissue and cells.^{12,15}

In our previous study evaluating the relationship between miR-451a and MIF,¹² we reported that miR-451a was elevated in endometriotic lesion tissue compared to matched eutopic endometrium from women with endometriosis. In that study, it was demonstrated that miR-451a expression was associated with endometriotic lesion survival. Based on this observation, coupled with the strong supportive evidence that miR-451a modulates cell proliferation/survival, the goal of this project was to evaluate whether serum levels correlated with miR-451a lesion content and whether miR-451a serum levels were elevated in women with confirmed endometriosis.

Materials and Methods

Human Participants and Tissue Acquisition

The study was approved by the institutional review boards of both University of Kansas Medical Center and Cleveland Clinic. Written informed consent was obtained prior to surgical removal of endometriotic lesion tissue and endometrial biopsies. Samples were obtained from a total of 81 women (N = 81) between the ages of 21 and 45. Women with endometriosis who presented with pelvic pain due to failed previous endometriosis treatment and were undergoing surgical removal of endometriotic lesion tissue were enrolled. A total of 41 participants were enrolled (n = 16 in the proliferative stage of the menstrual cycle and n = 25 in the secretory stage of the menstrual cycle) and included women with stage I/II (n = 12) and stage III/IV (n = 29) endometriosis (Table 1). No participants had taken gonadotropin-releasing hormone (GnRH) analogs or hormonal therapies within 3 months prior to surgery. A total of 41 endometrial biopsies (eutopic endometrium) and 41 matched (the same patient) endometriotic lesions were collected. All specimens were collected by the same surgeon (T.F.) at Cleveland Clinic with emphasis on minimizing sample contamination from underlying/surrounding nonendometriotic lesion tissue. To do so, endometriotic lesions were excised and sent to pathology for confirmation of endometriosis, which was defined as the presence of endometrial glands and stroma. Tissue was excised using sharp scissors with no energy. During the excision, the underlying tissue was separated from the lesion

Table 1. Patient Demographics in Control and Endometriosis Groups.

Study Group	Age Range
Control (N = 40)	
No symptoms ^a (n = 20)	21-38
No signs ^b (n = 20)	22-45
Endometriosis (N = 41)	
Stage I/II; peritoneal disease only (n = 12)	23-40
Stage III/IV; peritoneal disease only (n = 23)	26-44
Peritoneal and ovarian endometrioma (n = 6)	

^aSubjects exhibiting normal menstrual cycles, no symptoms of endometriosis including pain or infertility, not undergoing surgical procedures for evaluation of pelvic cavity.

^bSubjects undergoing surgical procedure for diagnostic laparoscopy for pain (n = 18) or pain and bleeding (n = 2).

tissue. A portion of the same sample lesion which was sent for endometriosis confirmation by a pathologist was utilized for research. Research samples were immediately snap frozen, stored at -80°C and then shipped to the University of Kansas Medical Center.

Controls consisted of women derived from 2 groups. The first group of participants (n = 20) consisted of women undergoing exploratory laparoscopy for pain or pain with bleeding in which no visible signs of endometriosis were found, whereas the second group (n = 20) of women consisted of healthy volunteers with no visible signs and/or symptoms of pelvic pain or infertility (purchased from Innovative Research, Novi, Michigan). Patient demographics for all groups are summarized in Table 1, and no significant differences among the ages of patients in each group were detected. Women in neither control group had current or a history of autoimmune diseases.

The stage of the menstrual cycle was determined from the patient's medical records and/or assessment of serum progesterone values with the proliferative stage defined as serum progesterone concentrations ≤ 2.0 ng/mL and the secretory stage defined as serum progesterone concentrations > 2.0 ng/mL as established in the literature. Samples were subjected to RNA extraction followed by quantitative real-time polymerase chain reaction (qRT-PCR) analysis as described below. As no difference in miR-451a expression was noted among stages of the menstrual cycle, stages of endometriosis, or influenced by medications, data were collapsed and analyzed as ectopic versus eutopic tissue for miR-451a expression.

Baboon Model of Endometriosis and Sample Collection

All animal studies were approved by the Animal Care and Use Committee of the University of Illinois at Chicago and Michigan State University. Experimental endometriosis was induced in baboons (N = 8) as previously described.²³ Briefly, baboons received intraperitoneal inoculation with menstrual endometrium on day 2 of 2 consecutive menstrual cycles (day 1 = the first day of menses). Blood samples were obtained prior to the

first inoculation and then at 1, 3, 6–7, 9–10, and 15–16 months after inoculation. Samples were processed for serum separation, and aliquoted serum samples were stored at -80°C until shipped to the University of Kansas Medical Center for RNA isolation and miR-451a assessment ($N = 8$ for each time point except 3 months where $n = 4$). The progression of disease was monitored and endometriosis was confirmed and staged by laparotomy in each animal by consecutive laparoscopies and video recording at each of the time points following inoculation.

Total RNA Extraction From Serum and miRNA qRT-PCR Analysis

Patient serum samples were prepared following the same standard centrifugation and sample preparation steps from all institutions providing samples. RNA was isolated from 0.5 mL of serum using an equal volume of TRIzol (Life Technologies, Carlsbad, CA), whereas tissue RNA was extracted using 1.0 mL of TRIzol/100 mg of tissue following the protocols provided by the manufacturer. To assess miR-451a expression, miRNA kits for miR-451a were purchased from Applied Biosystems (Foster City, CA). Total RNA (250 ng in 5 μL) was reverse transcribed using RT kits following the manufacturer's protocol with the following modifications. Briefly, miRNAs were reverse transcribed in a single reaction using 2 μL of each miRNA-specific 5X RT primers. The resulting material was then used for independent qRT-PCR for each miRNA. To normalize for starting material, a reverse snRNA U6 was included in the miRNA RT reactions, and qRT-PCR of U6 was performed using previously validated primers²⁴ with forward and reverse sequences—U6 forward: CTCGCTTCGGCAGCAC and U6 reverse: AACGCTTCACGAATTTGCGT. Quantitative real-time polymerase chain reaction reactions were completed on a 7900 HT Sequence Detection System (Applied Biosystems). All samples were run in triplicate and the average value was used in subsequent calculations. The $2^{-\Delta\Delta\text{CT}}$ method was used to calculate the fold-change values among samples as previously described by our group.^{14,25} For qRT-PCR assays, the intra- and interassay coefficients of variation were both less than 5%.

Serum Progesterone Analysis

Serum progesterone concentrations were measured by enzyme-linked immunosorbent assay in human samples following the recommendations of the manufacturer (Abnova, Walnut, California). The sensitivity of the assay is 8.57 pg/mL and the intra- and interassay coefficients of variation were approximately 5.97% and 5.93%, respectively. All samples were assessed in duplicate.

Statistical Analysis

Serum miR-451a levels were first separately assessed within stages of endometriosis (stage I/II vs stage III/IV in

Figure 1. Correlation between endometriotic lesion and sera miR-451a levels. Pearson correlation coefficient (r) was calculated as described under “Materials and Methods.” A significant positive correlation was noted ($r = 0.4279$) between the 2 parameters. miR-451a indicates microRNA-451a.

endometriosis participants) and between the 2 control groups—women with pain but no visible signs of endometriosis and women with no symptoms of endometriosis (pain or infertility). As no significant difference in miR-451a expression could be attributed to stages of endometriosis or between the 2 control groups, data were pooled and analyzed as endometriosis compared to a single control group with separation based on stages of menstrual cycle within each study group. All data were first assessed for normal (Gaussian) distribution. Both human and baboon data failed to display normality of distribution and were therefore analyzed by nonparametric tests as specified below. Differences in the ΔCt values among participant groups or among time points (baboon sera across different times post-endometriosis induction) were analyzed using Kruskal-Wallis test followed by post hoc analysis using Dunn multiple comparison test. The diagnostic performance of miR-451a expression levels was assessed using a receiver operating curve (ROC) to plot the test sensitivity versus its false-positive rate (1-specificity). To examine the correlation between serum miR-451a and lesion miR-451a content, Spearman correlation coefficients were calculated. All analyses were conducted using GraphPad Prism version 6. The significance was set at $P < .05$ for all analyses.

Results

To examine the relationship between serum miR-451a and endometriotic lesion miR-451a content, Spearman correlation coefficients were calculated. Endometriotic lesion miR-451a content displayed a positive correlation with serum miR-451a content (Figure 1, $r = 0.4279$; 95% confidence interval [CI] = 0.1292–0.6554; $P = .0053$). Initial assessment of serum

Figure 2. Sera miR-451a levels are elevated in women with endometriosis. Sera miR-451a levels were assessed in women with ($n = 41$) and without ($n = 40$) endometriosis during the proliferative ($n = 34$) and secretory stages ($n = 47$) as described in “Materials and Methods”. Data were analyzed as control versus endometriosis for miR-451a expression during each stage of the menstrual cycle. A, ΔCt values among groups were analyzed by Kruskal-Wallis test followed by post hoc analysis using Dunn multiple comparison test. $**P < .001$ and $*P < .01$ between control and endometriosis during the proliferative and secretory stages of the menstrual cycle, respectively. Please note that lower ΔCt values correspond to an increase in miR-451a expression. B, Sera miR-451a levels were approximately 25-fold higher in endometriosis samples compared to controls and was calculated using the formula $2^{(x)}$ where $(x) = (\Delta Ct \text{ control} - \Delta Ct \text{ endometriosis})$. miR-451a indicates microRNA-451a.

miR-451a levels in control and participants with endometriosis based on the presence/absence of pain or stages of endometriosis, respectively, did not differ (data not shown). Therefore, we pooled data within each group and compared serum miR-451a levels between control and participants with endometriosis taking into account stages of menstrual cycle. As displayed in Figure 2A, serum from participants with endometriosis exhibited significantly lower miR-451a ΔCt values compared to controls. This was true for comparisons between both the proliferative and secretory stages between study groups (Figure 2A). These ΔCt values corresponded to an approximate 40-fold increase during the proliferative stage of the menstrual cycle within the endometriosis group compared to controls and an approximate 23-fold increase during the secretory stage (Figure 2B).

To assess the utility of circulating miR-451a in the potential diagnosis of endometriosis, we examined the ROC curve of

Figure 3. Receiver operating characteristic (ROC) curve for miR-451a in women with and without endometriosis. An ROC curve was constructed as described under “Materials and Methods” and showed an area under the curve (AUC) of 0.8599. miR-451a indicates microRNA-451a.

serum miR-451a (Figure 3), which was differentially expressed between endometriosis patients and controls. The area under the curve (AUC) of miR-451a was 0.8599 (95% CI = 0.7780–0.9421). Using a ΔCt value of <3.495 , sensitivity and specificity were 85.37% (95% CI = 70.83%–94.43%) and 84.62% (95% CI = 69.47%–94.14%), respectively.

To begin to determine why miR-451a sera levels may be elevated in women with endometriosis, we assessed serum miR-451a levels in baboons before and after the induction of experimental endometriosis. Sera ΔCt values were highly variable both within and between baboons. Statistical analysis revealed a significantly lower ΔCt value (which corresponds to a higher serum miR-451a level) at 6 months postinoculation compared to preinoculation values (Figure 4A). This change was statistically significant and corresponds to an approximate 20-fold increase in sera miR-451a levels at 6 months post-endometriosis induction (Figure 4B). Interestingly, in those baboons which showed more minimal changes at 6 months postinoculation compared to postinduction values, their sera miR-451a levels were further increased at 9 months postinoculation compared to levels at 6 months (data not shown). As can be seen from Figure 4A, the general trend is that ΔCt values decreased (sera miR-451a levels increased) after induction of endometriosis, reaching peak levels at 6 months postinduction. Levels were most variable at 3 months postinduction, reached significantly higher levels in the serum at 6 months postinduction, and then became variable at 9 and 15 months postinduction (Figure 4). Overall, induction of endometriosis was associated with an increase in circulating miR-451a levels compared to sera levels from baboons obtained prior to induction of endometriosis, demonstrating that the elevated circulating levels of miR-451a are a result of the disease/presence of ectopic lesions.

Figure 4. miR-451a sera levels in baboons pre- and post-endometriosis induction. Blood was collected and serum was isolated from baboons prior to endometriosis induction (PI) and then again at 1, 3, 6, 9–10, and 15–16 months PI (N = 8 for all time points but 3 months were n = 4). Sera miR-451a levels were assessed by qRT-PCR as described in “Materials and Methods”. Δ Ct values among the time points were analyzed by Kruskal-Wallis test followed by Dunn test. Different letters indicate statistical significance ($P < .05$) among the means. A, Data are displayed as the mean $\pm$ SEM for Δ Ct values. B, Data are displayed as average fold change in sera miR-451a values for each month postinduction compared to PI values using the formula, $2^{(x)}$ where $(x) = (\Delta$ Ct PI – Δ Ct for each specified time point). miR-451a indicates microRNA-451a; PI, postinduction; qRT-PCR, quantitative real time polymerase chain reaction; SEM, standard error of the mean.

Discussion

The primary objective of the current study was to follow up our initial report that miR-451a expression is elevated in endometriotic lesion tissue compared to the corresponding eutopic endometrium, to determine the relationship between lesion and sera content in these women, as well as to begin to explore the reason for elevated sera miR-451a levels. MicroRNA-451a is expressed in the circulation, predominantly by erythrocytes,²⁶ where it is proposed to play a role in the differentiation and/or maturation of red blood cells.²⁷ We detected a significant difference between sera levels in women with endometriosis compared to women without visible signs of the disease and/or symptoms of the disease. The positive correlation detected between lesion and serum miR-451a suggests that the later may be derived from lesion tissue itself or may be manifested as a result of the presence of ectopic tissue. Using the well-described baboon model for endometriosis, we provide additional support that the presence of ectopic lesions is associated

with elevated sera levels of miR-451a suggesting that the elevated levels of circulating miR-451a are a result of the disease and perhaps not a causative factor in its development. Due to limitations in the current study (unavailability of baboon lesions for miR-451a assessment), we were unable to assess the relationship between lesion and serum content as endometriosis progressed (from 3 to 15 months postinduction) in these animals.

The observation that sera miR-451a levels correlated with lesion content lead us to examine whether miR-451a circulating levels could distinguish between women with visible signs/symptoms of endometriosis from those women who do not exhibit endometriosis symptoms (primarily pain and infertility). We found a significant elevation in serum miR-451a levels in women with confirmed endometriosis compared to women who exhibited pain but no visible signs of endometriosis as well as women who exhibited neither pain nor symptoms of endometriosis. This observation is in agreement with a recent report by Cosar and colleagues²⁸ who utilized a comprehensive array-based analysis of serum miRNAs and identified several miRNAs in serum that distinguished participants with endometriosis from those without. Of these, miR-125b-5p had the greatest potential as a single diagnostic biomarker and in combination with miR-451a and miR-3613-5p further improved diagnostic performance. The AUC and 95% CI for miR-451a in the study by Cosar and colleagues²⁸ were 0.835 and 0.707 to 0.963, respectively, whereas our AUC was 0.8599 and 95% CI was 0.778 to 0.942, thus demonstrating in 2 independent populations the potential utility of using miR-451a levels to distinguish between women with and without endometriosis.

Area under the curve values provide a measure of discrimination or ability of the test to correctly classify those with and without the disease, and based on our results in this study and those of Cosar and colleagues,²⁸ serum miR-451a values would be able to correctly classify the presence/absence of endometriosis approximately 85% of the time. Furthermore, for miR-451a to serve as a useful diagnostic marker, specificity and sensitivity must be evaluated. Sensitivity, or the true positive rate, measures the proportion of actual positives which are correctly identified as positive for the presence of a disease (endometriosis in this case). Specificity, or the true negative rate, measures the proportion of negatives which are correctly identified as negative for the presence of a disease (endometriosis). For a “perfect” diagnostic marker, both sensitivity and specificity would be 100%. Using a Δ Ct value of less than 3.495, sensitivity and specificity were calculated as 85.37% and 84.62%, respectively, for serum miR-451a.

The issue of sensitivity and specificity has been one of the limiting factors in identifying a diagnostic marker for endometriosis. Cancer antigen 125 (CA125) was one of the first potential biomarkers to be evaluated for endometriosis diagnosis.^{29–32} However, CA125 has poor specificity and sensitivity as elevated CA125 levels are detected in pregnant females,^{33,34} other gynecological pathologies,³⁵ and pelvic inflammatory disorders.³⁶ More recent emphasis has focused

on the identification of panels of biomarkers for endometriosis diagnosis which, when assessed in combination, offer superior specificity and sensitivity compared to the assessment of these markers singularly.^{37,38} Using this approach, the issues of specificity and sensitivity have been greatly improved over the utilization of single markers such as CA125.

MicroRNAs may hold promise as circulating biomarkers as they are resistant to RNase degradation and as such are stable within biological fluids.³⁹ Several miRNAs have been evaluated as potential diagnostic markers for endometriosis in human sera^{24,28,40,41} and plasma.⁴²⁻⁴⁴ Cho and colleagues²⁴ demonstrated that several circulating miRNAs were differentially expressed in the sera of women with endometriosis compared with controls. Furthermore, they demonstrated that the combination of serum let-7b, -7d, and -7f levels during the proliferative phase may serve as a diagnostic marker for endometriosis with a maximum AUC score of 0.929. Wang and colleagues⁴⁰ found a total of 51 serum miRNAs to be either downregulated or upregulated 10-fold or greater in sera from women with endometriosis. From these differentially expressed miRNAs, miR-122 and miR-199a were shown to be upregulated and miR-145, miR-141*, miR-542-3p, and miR-9* were downregulated. Furthermore, miR-199a and miR-122 were also differentially expressed between severe and mild endometriosis with their expression showing progressive changes with the severity of the disease. The diagnostic value of these molecules was confirmed with the optimal combination of miR-199a, miR-122, miR-145*, and miR-542-3p yielding an AUC of 0.994 and a cutoff point (0.4950) of 93.22% sensitivity and 96.00% specificity. However, Hsu and colleagues reported that sera miR-199a-5p levels were downregulated in endometriosis,⁴¹ suggesting some degree of uncertainty to the value of miR-199a as a potential diagnostic marker. Nonetheless, the AUC of 0.994 for the group of miRNAs in the study by Wang and colleagues has given the highest AUC of sera miRNAs to date.

Assessment of plasma levels of miRNAs has also identified potential biomarkers for endometriosis. Jia and colleagues⁴² demonstrated that miR-17-5p, miR-20a, and miR-22 were significantly lower in plasma from women with endometriosis compared to controls, yielding an AUC of 0.74, 0.79, and 0.85, respectively. A study by Suryawanshi and colleagues⁴³ evaluated plasma miRNAs levels in women with endometriosis compared to women with endometriosis-associated ovarian cancer and controls. Of 24 candidate miRNAs, only miR1978 and miR-362-5p were not elevated in plasma from women with endometriosis-associated ovarian cancer compared to controls and could possibly be used to distinguish participants with endometriosis from the other groups. Finally, miR-200a and miR-141 have also been identified as potential sera biomarkers for endometriosis.⁴⁴ Interestingly, in this study, it was found that the plasma miR-200a, miR-200b, and miR-141 levels vary with time of blood sampling which would indicate that time of collection would need to be standardized when collecting blood for miRNA analysis.

Compared to the miRNAs assessed to date, miR-451a appears to be as good as, or superior to, those potential miRNA diagnostic markers proposed in other studies. However, the utility of miR-451a as an endometriosis biomarker is not without limitation. MicroRNA-451a levels have been shown to be differentially expressed in patients with nonalcoholic fatty liver disease,⁴⁵ rheumatoid arthritis (RA),⁴⁶ and several types of cancer including renal cell carcinoma,⁴⁷ nonsmall lung cancer,⁴⁸ and epithelial ovarian cancer.⁴⁹ Although our data demonstrate a strong ability to discriminate between women with and without endometriosis based on miR-451a serum levels, we must remember that this miRNA is associated with other diseases, namely of autoimmune nature, which share similarities with endometriosis.^{50,51} The potential of other conditions and/or diseases in concurrence with endometriosis must be fully considered when assessing serum miR-451a levels and its potential diagnostic utility. The best application of miR-451a as a diagnostic marker for endometriosis may come from the development of a panel of differentially expressed miRNAs such as those described earlier in the text^{24,28,40-44} which may increase specificity and sensitivity.

In addition, defining the mechanism which leads to elevated serum miR-451a levels may also aid in the development of the most specific diagnostic tests for endometriosis. As we demonstrate in the current study, there is a positive correlation between serum miR-451a and endometriotic lesion 451a content which may suggest that the elevated circulatory levels may be derived at least in part from lesion-derived miR-451a. As serum of normal (nonendometriosis) women contain miR-451a, it is likely that other cellular sources contribute to circulating miR-451a levels and that the endometriotic lesions may provide additional “enrichment” of these levels. We have previously demonstrated in women with endometriosis that ectopic lesions express miR-451a and that this expression is significantly higher compared to matched eutopic endometrial expression.¹² This pattern of expression was also detected in baboons with experimental endometriosis as miR-451a expression was greater in ectopic lesions (15 months postinduction) compared to matched eutopic endometrium.¹⁵

We did not detect a difference in lesion¹² or sera miR-451a levels based on stages of endometriosis (I/II vs III/IV). These observations may suggest that changes in sera miR-451a are manifested early in the disease process/endometriosis development and may not be influenced by factors associated with the more severe forms of the disease such as pelvic adhesion, fibrosis, and excessive disease.

We are aware, and also propose, that the hormonal milieu generated by endometriosis could contribute to the elevated circulating miR-451a levels. It is well-established that endometriosis is associated with an inflammatory environment.^{52,53} Elevated levels of miR-451a are positively correlated with RA T-cell DAS28, ESR levels, and with serum concentrations of IL-6.⁵⁴ Interestingly, after treatment with methotrexate and anti-TNF- α drugs, the levels of miR-451 in T cells significantly decreased as compared to cells from newly diagnosed patients with active RA.⁵⁴ From this information, we propose

that the most likely explanation may lie somewhere between miR-451a levels derived from the lesion itself and other cellular sources which contribute to the circulating levels of this miRNA in women with endometriosis.

Finally, we are aware that this study has limitations. For example, we are aware that the control group which consisted of healthy women who exhibited no signs of pelvic pain or infertility could have microscopic endometriosis. If these women were to have endometriosis, the rationale for treatment would be uncertain as they would apparently exhibit neither of the 2 major symptoms of the disease—pain and infertility. Second, this was a retrospective study and to fully evaluate the potential diagnostic utility of miR-451a, or any diagnostic marker, a larger prospective study is warranted, which is currently underway. We conclude that serum miR-451a levels are positively correlated with endometriotic lesion miR-451a content in women with endometriosis and that circulating miR-451a levels may serve as a potential diagnostic aid in the detection of endometriosis.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was funded by the National Institutes of Health/NICHD by grant NIH HD069043 and HD073733 to W.B.N. and NIH HD082453 and HD083273 to A.T.F.

References

- Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364(9447):1789-1799.
- Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM. Retrograde menstruation in healthy women and in patients with endometriosis. *Obstet Gynecol*. 1984;64(2):151-154.
- Liu DT, Hitchcock A. Endometriosis: its association with retrograde menstruation, dysmenorrhoea and tubal pathology. *Br J Obstet Gynaecol*. 1986;93(8):859-862.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.
- Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science*. 2007;318(5858):1931-1934.
- Erson AE, Petty EM. MicroRNAs in development and disease. *Clin Genet*. 2008;74(4):296-306.
- Ohlsson Teague EM, Van der Hoek KH, Van der Hoek MB, et al. MicroRNA-regulated pathways associated with endometriosis. *Mol Endocrinol*. 2009;23(2):265-275.
- Filigheddu N, Gregnanin I, Proporato PE, et al. Differential expression of microRNAs between eutopic and ectopic endometrium in ovarian endometriosis. *J Biomed Biotechnol*. 2010;2010:369549.
- Hawkins SM, Creighton CJ, Han DY, et al. Functional microRNA involved in endometriosis. *Mol Endocrinol*. 2011;25(5):821-832.
- Braza-Boïls A, Mari-Alexandre J, Gilabert J, et al. MicroRNA expression profile in endometriosis: its relation to angiogenesis and fibrinolytic factors. *Hum Reprod*. 2014;29(5):978-988.
- Shi XY, Gu L, Chen J, Guo XR, Shi YL. Downregulation of miR-183 inhibits apoptosis and enhances the invasive potential of endometrial stromal cells in endometriosis. *Int J Mol Med*. 2014;33(1):59-67.
- Graham A, Falcone T, Nothnick WB. The expression of microRNA-451 in human endometriotic lesions is inversely related to that of macrophage migration inhibitory factor (MIF) and regulates MIF expression and modulation of epithelial cell survival. *Hum Reprod*. 2015;30(3):642-652.
- Bandres E, Bitarte N, Arias F, et al. microRNA-451 regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells. *Clin Cancer Res*. 2009;15(7):2281-2290.
- Liu G, Xu Z, Hao D. MicroRNA-451 inhibits neuroblastoma proliferation, invasion and migration by targeting macrophage migration inhibitory factor. *Mol Med Rep*. 2016;13(3):2253-2260.
- Joshi NR, Su RW, Chandramouli GV, et al. Altered expression of microRNA-451 in eutopic endometrium of baboons (*Papio anubis*) with endometriosis. *Hum Reprod*. 2015;30(12):2881-2891.
- Wang R, Wang ZX, Yang JS, Pan X, De W, Chen LB. MicroRNA-451 functions as a tumor suppressor in human non-small cell lung cancer by targeting ras-related protein 14 (RAB14). *Oncogene*. 2011;30(23):2644-2658.
- Zhang T, Sun Q, Liu T, et al. MiR-451 increases radiosensitivity of nasopharyngeal carcinoma cells by targeting ras-related protein 14 (RAB14). *Tumour Biol*. 2014;35(12):12593-12599.
- Tian Y, Nan Y, Han L, et al. MicroRNA miR-451 downregulates the PI3K/AKT pathway through CAB39 in human glioma. *Int J Oncol*. 2012;40(4):1105-1112.
- Zang WQ, Yang X, Wang T, et al. MiR-451 inhibits proliferation of esophageal carcinoma cell line EC9706 by targeting CDKN2D and MAP3K1. *World J Gastroenterol*. 2015;21(19):5867-5876.
- Yin P, Peng R, Peng H, et al. MiR-451 suppresses cell proliferation and metastasis in A549 lung cancer cells. *Mol Biotechnol*. 2015;57(1):1-11.
- Zhu S, Huang Y, Su X. Mir-451 correlates with prognosis of renal cell carcinoma patients and inhibits cellular proliferation of renal cell carcinoma. *Med Sci Monit*. 2016;22:183-190.
- American Society for Reproductive Medicine. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil Steril*. 1997;67(5):817-821.
- Hastings J, Fazleabas A. A baboon model for endometriosis: implications for fertility. *Reprod Biol Endocrinol*. 2006;4(suppl 1):S7.
- Cho S, Mutlu L, Grechukhina O, Taylor HS. Circulating microRNAs as potential biomarkers for endometriosis. *Fertil Steril*. 2015;103(5):1252-1260.
- Nothnick WB, Healy CA. Estrogen induces distinct patterns of microRNA expression within the mouse uterus. *Reprod Sci*. 2010;17(11):987-914.
- Rathjen T, Nicol C, McConkey G, Dalmay T. 2006. Analysis of short RNAs in the malaria parasite and its red blood cell host. *FEBS Lett*. 580(22):5185-5188.

27. Masaki S, Ohtsuka R, Abe Y, Muta K, Umemura T. Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. *Biochem Biophys Res Commun.* 2007;364(3):509-514.
28. Cosar E, Mamillapalli R, Ersoy GS, Cho S, Seifer B, Taylor HS. Serum microRNAs as diagnostic markers of endometriosis: a comprehensive array-based analysis. *Fertil Steril.* 2016;106(2):402-409.
29. Patton PE, Field CS, Harms RW, et al. CA-125 levels in endometriosis. *Fertil Steril.* 1986;45(6):770-773.
30. Giudice LC, Jacobs A, Pineda J, et al. Serum levels of CA-125 in patients with endometriosis: a preliminary report. *Fertil Steril.* 1986;45(6):876-878.
31. Pittaway DE, Faye JA. The use of CA-125 in the diagnosis and management of endometriosis. *Fertil Steril.* 1986;46(5):790-795.
32. Santulli P, Streuli I, Melonio I, et al. Increased serum cancer antigen-125 is a marker for severity of deep endometriosis. *J Minim Invasive Gynecol.* 2015;22(2):275-284.
33. Niloff JM, Knapp RC, Schaetzl E, et al. CA125 antigen levels in obstetric and gynecologic patients. *Obstet Gynecol.* 1984;64(5):703-707.
34. Sarandakou A, Protonotariou E, Rizos D. Tumor markers in biological fluids associated with pregnancy. *Crit Rev Clin Lab Sci.* 2007;44(2):151-178.
35. Niloff JM, Klug TL, Schaetzl E, et al. Elevation of serum CA125 in carcinomas of the fallopian tube, endometrium, and endocervix. *Am J Obstet Gynecol.* 1984;148(8):1057-1058.
36. Asher V, Hammond R, Duncan TJ. Pelvic mass associated with raised CA125 for benign condition: a case report. *World J Surg Oncol.* 2010;8:28.
37. Vodolazkaia A, El-Aalamat Popovic D, et al. Evaluation of a panel of 28 biomarkers for the non-invasive diagnosis of endometriosis. *Hum Reprod.* 2012;27(9):2698-2711.
38. Borrelli GM, Abrão MS, Mechsner S. Can chemokines be used as biomarkers for endometriosis? A systematic review. *Hum Reprod.* 2014;29(2):253-266.
39. Nothnick WB, Al-Hendy A, Lue JR. Circulating micro-RNAs as diagnostic biomarkers for endometriosis: privation and promise. *J Minim Invasive Gynecol.* 2015;22(5):719-726.
40. Wang WT, Zhao YN, Han BW, Hong SJ, Chen YQ. Circulating microRNAs identified in a genome-wide serum microRNA expression analysis as noninvasive biomarkers for endometriosis. *J Clin Endocrinol Metab.* 2013;98(1):281-289.
41. Hsu CY, Hsieh TH, Tsai CF, et al. miRNA-199a-5p regulates VEGFA in endometrial mesenchymal stem cells and contributes to the pathogenesis of endometriosis. *J Pathol.* 2014;232(3):330-343.
42. Jia SZ, Yang Y, Lang J, et al. Plasma miR-17-5p, miR-20a and miR-22 are down-regulated in women with endometriosis. *Hum Reprod.* 2013;28(2):322-330.
43. Suryawanshi S, Vlad AM, Lin HM, et al. Plasma microRNAs as novel biomarkers for endometriosis and endometriosis-associated ovarian cancer. *Clin Cancer Res.* 2013;19(5):1213-1224.
44. Rekker K, Saare M, Roost AM, et al. Circulating miR-200-family micro-RNAs have altered plasma levels in patients with endometriosis and vary with blood collection time. *Fertil Steril.* 2015;104(4):938-946.
45. Yamada H, Suzuki K, Ichino N, et al. Associations between circulating microRNAs (miR-21, miR-34a, miR-122 and miR-451) and non-alcoholic fatty liver. *Clin Chim Acta.* 2013;424:99-103.
46. Churov AV, Oleinik EK, Knip M. MicroRNAs in rheumatoid arthritis: altered expression and diagnostic potential. *Autoimmun Rev.* 2015;14(11):1029-1037.
47. Redova M, Poprach A, Nekvindova J, et al. Circulating miR-378 and miR-451 in serum are potential biomarkers for renal cell carcinoma. *J Transl Med.* 2012;10:55.
48. Markou A, Sourvinou I, Vorkas PA, Yousef GM, Lianidou E. Clinical evaluation of microRNA expression profiling in non-small cell lung cancer. *Lung Cancer.* 2013;81(3):388-396.
49. Ling S, Ruiqin M, Guohong Z, Ying W. Expression and prognostic significance of microRNA-451 in human epithelial ovarian cancer. *Eur J Gynaecol Oncol.* 2015;36(4):463-468.
50. Harris HR, Costenbader KH, Mu F, et al. Endometriosis and the risks of systemic lupus erythematosus and rheumatoid arthritis in the Nurses' Health Study II. *Ann Rheum Dis.* 2016;75(7):1279-1284.
51. Nielsen NM1, Jørgensen KT, Pedersen BV, Rostgaard K, Frisch M. The co-occurrence of endometriosis with multiple sclerosis, systemic lupus erythematosus and Sjogren syndrome. *Hum Reprod.* 2011;26(6):1555-1559.
52. Nothnick W, Alali Z. Recent advances in the understanding of endometriosis: the role of inflammatory mediators in disease pathogenesis and treatment. *F1000Res.* 2016;17:5.
53. Taylor RN, Kane MA, Sidell N. Pathogenesis of endometriosis: roles of retinoids and inflammatory pathways. *Semin Reprod Med.* 2015;33(4):246-256.
54. Smigielska-Czepiel K, van den Berg A, Jellema P, et al. Comprehensive analysis of miRNA expression in T-cell subsets of rheumatoid arthritis patients reveals defined signatures of naive and memory Tregs. *Genes Immun.* 2014;15(2):115-125.