Chapter

microRNA and Overcoming the Challenges of Their Use in the Diagnosis of Endometriosis

Victoria Turpin, Anna Leonova, Sanjay K. Agarwal and Warren G. Foster

Abstract

Endometriosis is a common estrogen dependent and inflammatory disease affecting approximately 176 million women worldwide. Currently, the time between onset of symptoms and a definitive diagnosis has been reported by several international studies to range from 6 to 12 years. Presently, laparoscopic surgery followed by histopathological confirmation of lesions remains the gold standard for diagnosis. In part because of cost and invasiveness, current trends favor reduced laparoscopic surgeries in preference of the non-surgical diagnosis of endometriosis. However, the search for a clinical marker or markers of endometriosis that provide equal or similar sensitivity and specificity to laparoscopy has remained elusive. Thus, the search for a diagnostic test for the diagnosis of endometriosis continues to be a high priority research and clinical issue. Recent studies have reported favorable results with microRNA; however, lack of replication and absence of validation suggest that circulating miRNA may not be reliable for clinical use. Use of different screening platforms together with divergent methods may account for some of the lack or reproducibility in the literature. Herein we critically assess the recent literature and explore sources for discrepant findings. We suggest that prospective studies using validated reference miRNA to normalize results together with improved study design may yet reveal a suitable diagnostic marker or panel of markers for the diagnosis of endometriosis.

Keywords: microRNA, miRNA, diagnosis, plasma, endometriosis

1. Background

Endometriosis is a common estrogen dependent and progesterone resistant disease of unknown cause characterized by growth of endometrial cells outside the uterine cavity [1]. It is estimated that 6–11% of all women are affected by endometriosis reaching an estimated 176 million women globally [2]. A chronic painful disease [3], endometriosis causes substantial health distress and interference with normal activities including work resulting in an average loss of 10.8 h/week from work [2] all leading to diminished quality of life (QOL) for affected women and their families. Chronic pelvic pain and infertility are common symptoms of endometriosis that bring women with this disease to seek medical attention. Approximately 71–87% of all women experiencing chronic pelvic pain and 50% of infertile women are diagnosed with endometriosis [4]. Thus, women with

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endometriosis report significant health distress and interference with normal activities including work and leisure time activities all leading to a deleterious effect upon women's social functioning, emotional well-being, employment, and vitality [5].

An important obstacle to the timely diagnosis and effective management of endometriosis is the lack of a simple diagnostic test. Diagnosis has been reported to be delayed by between 6 and 12 years with an average of 9 years from the onset of symptoms to definitive diagnosis [6]. Hence, identification of a clinical tool for the diagnosis of endometriosis has become a high priority research objective [2, 7, 8]. Health care providers and patients face a number of challenges in arriving at a diagnosis of endometriosis including: early age at onset of symptoms, normalization of pain by primary care providers, and suppression of symptoms through intermittent use of oral contraceptive pills [9]. Symptoms of endometriosis are shared with many other diseases including autoimmune diseases, cancer, irritable bowel syndrome (IBS), and musculoskeletal abnormalities. Therefore, an ideal biomarker of endometriosis must differentiate between endometriosis and other explanations for patient symptoms. In addition, clinical markers should be as minimally invasive as possible, affordable and convenient to use with the added benefit of providing insight into potential treatment response. Furthermore, the ideal biomarker must provide equivalent or similar outcome measures of sensitivity, specificity, positive, and negative predictive values to laparoscopy.

Currently, the gold standard for diagnosis remains visualization of endometriotic lesions typically by laparoscopy followed by histopathological confirmation of disease [10]. Current trends favoring the non-surgical diagnosis of endometriosis increase the pressure to identify novel clinical markers of endometriosis. Despite a plethora of biochemical differences in the peripheral circulation, peritoneal fluid, and endometrial tissues of women with endometriosis compared to healthy controls, no marker has been found with adequate sensitivity or specificity to challenge laparoscopy for the diagnosis of endometriosis whether used alone or in a panel of clinical markers [11–16]. However, reports of promising results have been brought forward in the literature from which epigenetic markers are potentially the most exciting.

2. Candidate clinical markers of endometriosis

Multiple gene and protein expression levels have been documented in women with endometriosis compared to controls; however, none have yielded reliable clinical markers of disease. Recent studies investigating the mechanisms controlling gene expression have produced promising results. Several histone modifications have been associated with endometriosis. For example, endometriotic stromal cells (ESC) have a lower global acetylation level of H3, and histone deacetylases 1 and 2 (HDAC1 and HDAC2) were upregulated compared to women without endometriosis [17]. Furthermore, histone deacetylase inhibitor (HDACI) treatment promoted apoptosis by reactivating the silenced chromatin [18]. G-protein-coupled estrogen receptor (GPER) expression and proliferation of endometriotic cells was inhibited by treatment with the HDACI's romidepsin and vorinostat [19]. These data suggest that histone modifications are involved in the pathophysiology of endometriosis and that HDACI's are promising agents for endometriosis treatment. However, use of histone markers in the diagnosis of endometriosis has yet to be explored.

Long-chain non-coding RNA (lnc-RNAs) are 200–100,000 bp RNA molecules which do not encode for protein, but are involved in transcriptional and post-transcriptional regulation of gene expression [20]. They are the most common non-coding RNAs and are involved in cell proliferation, differentiation, and apoptosis; all processes central in the pathobiology of endometriosis [21]. Some lnc-RNAs proposed as diagnostic markers of endometriosis include: H19 [22], CHL1-AS2

[23, 24], AC002454.1 [25], lncRNA SRA (steroid receptor RNA activator) [26], MALAT-1 [27], and LINC01279 [28]. Results of a recent study revealed the lnc-RNA are carried in circulating extracellular vesicles in women with endometriosis [29]. However, use of lnc-RNAs in the diagnosis of endometriosis has not been evaluated in a prospective study of women with symptoms suggestive of endometriosis with an independent validation step and thus their clinical utility remains uncertain.

Several recent studies have documented aberrant expression of multiple microRNAs (miRNAs) in the eutopic and ectopic endometrium of women with endometriosis [30–37]. miRNAs are short non-coding RNAs, 19–25 nucleotides long, that negatively regulate mRNA translation by repressing the protein translational machinery or degrading their target transcripts. Greater than 2000 mature human miRNA sequences have been identified and are thought to regulate approximately 50% of all protein coding genes. Multiple recent studies have documented differential microRNA (miRNA) expression in endometriotic tissues compared to eutopic endometrium of women with endometriosis and controls [33, 38–40]. miRNA are thought to hold promise as diagnostic biomarkers of disease because they are post-transcriptional regulators of gene expression that are stably expressed over time in bodily fluids and tissues [41]. Briefly, miRNA regulate protein expression through binding to and inhibiting the translation of mRNA transcripts into protein [42]. miRNAs are synthesized in the cytoplasm from nucleic hairpin intermediates (pre-miRNA) [43] which are then processed to yield mature miRNA that resist RNase degradation [41]. miRNA form an RNA-induced silencing complex (RISC) with Argonaute, Dicer, TAR RNA binding protein (TRBP) and protein activator of PKR (PACT) to post-transcriptionally regulate genes by binding to the 3′ region of the mRNA transcript and inhibiting translation [44].

In the early 2000s, several studies proposed that circulating levels of miRNA are differentially expressed in women with endometriosis compared to controls and thus could have diagnostic value [30, 31, 45]. Different methods including *in situ* hybridization, targeted RT-PCR and several different screening platforms including miRNA based microarrays, next generation sequencing and bio-informatics followed by RT-PCR validation have subsequently revealed a broad spectrum of miRNAs that are differentially expressed in women with endometriosis compared to control groups [29–31, 45–56]. However, to date, only the results for hsa-miR-451a [47, 48], 199a-5p [31, 54] and hsa-miR-141-3p [31, 49] have been successfully replicated in more than one study (**Table 1**). For the vast majority of miRNAs, differential expression has only been reported in a single study or the results for a few miRNAs have not been replicated by other investigators. For example, circulating levels of hsa-miR-145 were lower in women with endometriosis compared to controls [31] whereas hsa-miR-145 levels did not differ [47] or were higher in women with endometriosis compared to the control groups [50]. We postulate that divergent results may be the consequence of different screening platforms and technologies used to identify candidate miRNA markers of disease [57–59] and control group characteristics. Moreover, we suggest that different reference material used to quantify circulating miRNA levels are an additional source of variation.

While 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 Cp values of RNU6 are highly variable from miRNA Cp values [51, 60, 61]. 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 [51]. Furthermore, circulating levels of miR-16-5p are altered by inflammation and stress [62, 63] and thus we suggest that both RNU6 and miR-16-5p are not suitable for normalization of circulating miRNA levels in women with endometriosis.

Fluid	Case/control (N)	Cases	Controls	Reference miRNA	Cycle stage	Differentially expressed hsa-miR's	Citation
Serum	60/25	Stages I–IV	Symptomatic	RNU6	NS	↑**199a** and **122**, ↓9*, **145***, **141***, and 542-3p	[31]
Serum	24/24	Stage III and IV	Symptomatic	RNU6	P vs. S	↓let-7b, c, d, e, f (P) and 135a (S)	[46]
Serum	24/24	Stage III and IV	Symptomatic	RNU6	NS	↓3613-5p and 6755-3p ↑18a-5p, 125b-5p, 143-3p, 150-5p 342-3p, **451a** and 500a-3p	[47]
Serum	41/40	Stages I–IV	Symptomatic (n = 20) and Healthy (n = 20)	RNU6	NS	↑**451a**	[48]
Serum	30/20	Stages I–II	Infertile	cel-miR-39	ND	↓30c-5p, 127-3p, 99b-5p, 15b-5p, 20a-5p, and ↑424-3p, 185-5p	[52]
Serum	45/35	Stages I–IV	Symptomatic	RNU6	ND	↑**122** and **199a**	[54]
Serum	40/25	Stages I–IV	Healthy	18 s mRNA	ND	↓199a-5p	[55]
Plasma	23/23	Stage III and IV	Symptomatic	miR-16	ND	↓17-5p, 20a and 22	[30]
Plasma	61/30	Stages I–IV	Symptomatic (n = 35) and Healthy (n = 30)	miR-30e and 99a	NS	↓200a-3p and **141-3p**	[49]
Plasma	55/23	Stages I–IV	Symptomatic	miR-103-3p	ND	↑**145** (stages I and II), ↓31(stages I–IV)	[50]
Plasma	Variable	Stage I–IV	Symptomatic (n = 8–39) Healthy (n = 8)	RNU6 vs. miR-16 vs. miR-30b	NS	↓139-3p, 155 and 574-3p	[51]
Plasma	51/41	Stages I–IV	Symptomatic	miR-106a-5p, 199a-3p, 150-5p, 425-5p, 125a-5p, and 30e-5p	NS	↓miR154-5p and 378a-3p, ↑196b-5p and 33a-5p	[53]
Plasma	60/30	Stages I–IV	Symptomatic	miR-28-3p and 423-3p	S	*125b-5p, 28-5p, 29a-3p	[56]

Fluid	Case/control (N)	Cases	Controls	Reference miRNA	Cycle stage	Differentially expressed hsa-miR's	Citation
Plasma	33/20	NR	Healthy	miR-132	NR	↑15b, 16, 191, 195, 1973, 1979, and 4284	[45]
Plasma	6/4	Stages III–IV	Healthy	RNU6	NR	↓375, 27a-3p, and 30d-5p	[29]

miRNA in bold have been replicated by at least one other group of investigators.
S = significant effect of menstrual cycle stage, NS = not significant, ND = not determined, NR = not reported, differential miR expression was either increased (↑) or decreased (↓) in women with endometriosis compared to controls.
*Direction could not be ascertained from the published report.

Table 1.
Summary of miRNA's differentially expressed by microarray and RT-PCR in women with endometriosis (cases) compared to controls.

3. Effect of reference miRNA used to normalize results

While serum RNU6 has been widely used as the reference miRNA in prior endometriosis studies [29, 31, 46–48, 54], its levels have previously been reported to be unstable, unreliable, and a poor reference for miRNA since it is not processed or protected in the same way as miRNA [61, 63]. Therefore, we suggest that choice of reference miRNA can influence ability to detect significant differences and the direction of significant differences elicited. Previous studies report that hsa-miR-451a is upregulated in women with endometriosis compared to symptomatic controls [47] and compared to both symptomatic and asymptomatic (healthy) control groups [48]. Both prior studies employed RNU6 as a reference. While hsa-miR-451a has been found to act as a tumor suppressor [64, 65], it is also a marker of hemolysis [66] and thus we suggest that care should be employed to exclude samples with hemolysis before analysis. The miRNA ratio of hsa-miR-451a and hsa-miR-23a-3p has been employed by others [56, 67] to monitor for sample hemolysis. Therefore, we suggest that hsa-miR-451a has limited value as a candidate marker of endometriosis.

4. Effect of control group definition

Several studies have employed healthy women as their control population [29, 45, 48, 49, 51, 55], thus allowing circulating miRNA levels in women with endometriosis to be compared to symptomatic and asymptomatic healthy control populations. While the majority of previous reports employed symptomatic controls [30, 31, 46–51, 53, 54, 56], hsa-miR-16-5p [30, 51] RNU6 (the most common) reference material used to normalize miRNA expression [31, 46–48, 51, 54]; reference materials that are unsuitable for normalizing serum miRNA expression. In our experience, differential miRNA expression was dependent upon whether comparisons were made with asymptomatic compared to symptomatic controls. Therefore, we suggest that control group characteristics on the differential expression of candidate miRNA in women with endometriosis merits further investigation.

While, lack of replication, absence of validation of results, and poor sensitivity and specificity currently limit the value of miRNA as diagnostic markers of

endometriosis [51], we propose that usefulness of miRNA for the diagnosis of endometriosis cannot be evaluated without appropriate determination of appropriate reference miRNA.

5. Future directions

Although identification of clinical markers of endometriosis has long been sought, none has so far been suitable to displace laparoscopy as the gold standard for diagnosis. Endometriosis is a complex heterogenous disease with variable presentation whose symptoms are easily confused with other clinical problems. Since endometriosis is detectable with high frequency amongst asymptomatic women [68] surgical exclusion of disease in the control group is essential to prevent biasing results towards the null. Consequently, we suggest that control or reference group definition is important. Numerous prior studies reporting differential miRNA expression in women with endometriosis have employed asymptomatic women as their reference population [29, 45, 48, 49, 51, 55]. However, healthy women without symptoms of endometriosis and without evidence of endometriosis by laparoscopy (asymptomatic control) and symptomatic women without evidence of disease at the time of laparoscopy (symptomatic control) are functionally different, yet both groups continue to be employed as controls in contemporary studies. Results from our laboratory suggest that inclusion of asymptomatic controls can produce misleading results and thus speculate that restricting the control group to symptomatic controls in future studies may improve reproducibility of results. In addition to control group, we propose that the use of validated reference miRNA to normalize results also affects detection of levels of miRNA differentially expressed in women with endometriosis compared to controls.

Having identified candidate miRNA for the diagnosis of endometriosis it will be important to determine their relationship with pelvic pain as well as response to treatment. In the absence of this data the potential prognostic value of candidate markers of endometriosis remains uncertain. We also propose that future studies with robust sample size will be needed to clarify the relationship between circulating miRNA levels and menstrual cycle phase. Studies reporting menstrual cycle stage and circulating miRNA levels are thus far have produced equivocal results [31, 46–49, 51, 53, 56]. Furthermore, lesion type (endometrioma, peritoneal endometriosis, deep infiltrating endometriosis) are biologically distinct and thus a single clinical marker is unlikely to be dysregulated in all lesion types and thus a panel of markers may be more relevant. Furthermore, duration of disease and age of lesion may also present with functional differences. Therefore, discovery of clinical markers should describe the lesion types present in study participants. The influence of study participant age and body mass index are also important variables associated with pelvic pain and disease severity that are frequently not considered in analyses of clinical markers of endometriosis. Finally, the functional role of candidate markers in endometriosis has the potential to suggest therapeutic targets for additional research.

6. Summary and conclusions

Use of reference miRNA that may not be ideal for normalization of results may account for noted weaknesses in the literature. Use of validated reference miRNA markers and careful control of sample condition for potential confounders should improve study replication. Finally, although circulating miRNA levels have low variability in women with endometriosis, it will be necessary for discovery phases to include a large number of study participants to control for participant age,

DOI: http://dx.doi.org/10.5772/intechopen.91324

menstrual cycle stage, BMI, stage of disease, and type of lesions. Thus, we suggest that despite set-backs with reproducibility of results, it may be too soon to judge the diagnostic potential of miRNA.

Acknowledgements

Funding support for this project was provided by a research operating grant (MOP142230) from the Canadian Institutes of Health Research to WGF. The authors are grateful to Ms. Annette Bullen for her assistance in study participant recruitment, access to clinical data, and sample collection. The assistance of the Endo@Mac surgical team (Drs. Nick Leyland, Sarah Scatollon, and Dustin Costescu) with access to study participants, staging of disease, and collection of blood samples is greatly appreciated.

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