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Circulating microRNAs as diagnostic biomarkers for endometriosis: privation and promise

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Abstract

Endometriosis represents a major medical concern in women of reproductive age. One of the major hurdles in successful treatment of endometriosis that remains is the limitation in the process of timely disease diagnosis. A simple blood test for endometriosis-specific biomarkers would offer a timelier, accurate diagnosis for the disease allowing earlier treatment intervention. While there have been considerable efforts to identify such biomarkers, no clear choice for such non-invasive diagnostic tools have been identified. MicroRNAs are small non-coding RNAs which have been intensively evaluated as biomarkers for several diseases and may hold promise for endometriosis diagnosis. In this review, we highlight the need for non-invasive testing for endometriosis, discuss the potential use of miRNAs as diagnostic tools for this disease, and consider potential limitations in the use of these small RNA molecules as diagnostic markers for endometriosis.

Introduction

Endometriosis is a chronic, recurrent disease in women of reproductive age. Endometriosis can present in patients with symptomatology such as cyclic pelvic pain, dysmenorrhea, dyspareunia or dyskinesia, and symptoms that are nonspecific and do not correlate with the extent or severity of the physical disease. It is estimated that up to 20 percent of women with endometriosis have concurrent chronic pelvic conditions including irritable bowel syndrome, interstitial cystitis/painful bladder syndrome, fibromyalgia, and migraines (1). Endometriosis has been estimated to be the third leading cause of gynecologic hospitalizations in the United States (2). In current practice, the disease can only be diagnosed by invasive procedures which themselves are associated with morbidities and limitations. A simple blood test would offer multiple benefits over the current diagnostic approach, but to date, no “endometriosis-specific” biomarkers have been identified. MicroRNAs (miRNAs) are small non-coding RNA molecules which possess superior

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stability in biological fluids and offer multiple benefits as potential diagnostic markers compared to current peptide/protein based tests in general. The potential utility of miRNAs as diagnostic markers for non-gynecological diseases as well as cancer has recently gained considerable attention.

The need for endometriosis diagnostic biomarkers

Endometriosis is defined as the presence of ectopic endometrial glands and stroma outside of the uterine cavity with development primary in the pelvic cavity. Endometriosis affects 5% to 10% of women of reproductive age and is characterized primarily by pelvic pain and infertility (3). In those women who experience painful periods, the prevalence escalates to 25 to 45% (4), while in women with infertility/subfertility, the prevalence ranges from 20% to 40% (5). Endometriosis affects a woman's quality of life by having a negative impact on normal activities such as career, sexual life, and fertility potential. The medical and economic burdens of endometriosis are further magnified by the cost of diagnosis, therapy, repeated surgeries for recurrent disease (3), and the possible association with ovarian and breast malignancies (6).

Diagnostic laparoscopy is the "gold standard" for endometriosis diagnosis but laparoscopy is not without drawbacks. Laparoscopy is an invasive procedure and as such requires general anesthesia and surgical skill. Further, potential hazards associated with the surgical procedure include major blood vessel or bowel injury (7). Limitations of diagnosis via visual inspection of the pelvic cavity may impact the ability to diagnose retroperitoneal and/or deep infiltrating lesions (8), it is also heavily operator dependent (9 – 11). Thus, as diagnostic laparoscopy remains the only universally accepted method of choice for endometriosis diagnosis; a simple blood test for the diagnosis of endometriosis would overcome these problems and have a major positive impact on women's health.

In addition to the drawbacks and limitations associated with endometriosis diagnosis by laparoscopy, additional concerns lie within the time to diagnosis of the disease. This delay to diagnosis has economic impacts on the patient and society as a whole as well as psychological impact on the patient. This may be due multiple reasons including non-specific symptoms of the disease (pelvic pain and infertility) which leads to multiple tests for differential diagnosis. Ultimately, endometriosis is confirmed by invasive means such as laparoscopy allowing visualization of the peritoneal cavity and confirmation of the presence of ectopic endometriotic implant tissue. As such, the identification of specific biomarkers for the disease which may allow earlier detection and may require less invasive diagnostic measures would reduce not only the time to diagnosis but also reduce the cost associated with performing laparoscopy and eventual diagnosis of the disease. Notwithstanding, laparoscopy remains that standard approach for endometriosis diagnosis.

The current state of endometriosis diagnostic biomarkers

Diagnostic markers are biological parameters that aid in the diagnosis of an existing disease, thus they should be specific to that disease and not show lack of specificity. Sensitivity, which is also referred to as the true positive rate, measures the proportion of actual positives which are correctly identified as positive for presence of a disease/condition. Specificity,

which is also referred to as the true negative rate, measures the proportion of negatives which are correctly identified as negative for the presence of a disease/condition. Ideally, a perfect diagnostic biomarker would exhibit 100% sensitivity and 100% specificity. However, in the real world, this is rarely the case and acceptable levels of trade-off between sensitivity and specificity must be reached.

The issue of sensitivity and specificity has been perhaps the major limiting factor in identifying an “endometriosis diagnostic marker.” Cancer antigen 125 (CA125) was one of, if not, the first biomarker evaluated for diagnosis of endometriosis (12 – 14), but this was not without question (13). First described as a biomarker for epithelial ovarian cancer (15), CA125 levels may still be considered when aiding in assessment of endometriosis severity (16), but this marker is clearly not specific for the disease. Like most putative endometriosis diagnostic biomarkers, CA125 suffers from poor sensitivity and specificity as elevated levels are detected in other gynecological pathologies (17), pregnancy (18) and pelvic inflammatory disorders (19).

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 assessment of these markers singularly (20, 21). Using this approach, the issues of specificity and sensitivity have been greatly improved over utilization of single markers such as CA125. While the approach of using panels of inflammatory and non-inflammatory markers (20, 21) appear to hold great promise many of these markers are not specific to the endometriotic lesions themselves.

MicroRNAs (miRNAs) have emerged as potential diagnostic markers for endometriosis based upon the premise of identification of endometriosis-specific miRNAs, following suit to the field of cancer diagnostic biomarkers. Much of the initial work on miRNAs as diagnostic markers come from the field of cancer biology as several reports in the literature have identified oncomiRs or miRNAs which are specific to tumor tissue. Thus, the potential of endometriotic lesion specific miRNAs would in theory provide greater specificity and sensitivity compared to current biomarkers or panels of biomarkers. In the following pages, we provide background on miRNAs, their profiles in endometriotic tissue and our current understanding if these small RNAs can be used as diagnostic and/or prognostic markers for endometriosis.

MicroRNA biogenesis and function

miRNAs are a class of small non-coding regulatory RNAs that regulate gene expression post-transcriptionally impacting subsequent translation of protein (22, 23). miRNAs are vital for normal development and function of essentially all organs and alterations in their normal pattern of expression have been associated with numerous diseases of these organ systems. Genes for specific miRNAs are primarily intergenic, residing between genes, and intronic, residing within introns. Less common are miRNA genes which have been shown to make up entire introns as well as reside within exons (24, 25). miRNA transcription is mediated by polymerase II (or III) as the initial long primary RNA transcript (pri-miRNA) is capped (MGpppG) and polyadenylated (26). Beginning in the nucleus, miRNA transcription begins

with transcription of the pri-miRNA transcript which then binds with the RNA-binding protein DGCR8 (DiGeorge syndrome critical region 8). The pri-miRNA-DGCR8 complex is then cleaved by Drosha (an RNase III enzyme). This cleavage yields a stem-loop precursor miRNA (pre-miRNA). An alternative pathway has also been described by which pre-miRNA hairpins can be generated by the lariat debranching enzyme, Ldbr, in the Drosha-independent mitron pathway (27, 28). Regardless of pathway, upon liberation, pre-miRNAs are then exported from the nucleus via exportin 5 and RAN-GTP into the cytoplasm. Once in the cytoplasm, a second RNase III enzyme, Dicer, cleaves the pre-miRNA into a transient RNA duplex which results in the generation of two strands. One of the two strands (deactivated strand) accumulates in P-bodies where they are either stored or degraded (29). The remaining strand (guide strand which is the mature miRNA) is preferentially loaded onto the RNA-induced silencing complex (RISC) which is composed of Dicer, a TAR RNA-binding protein (referred to as TRBP) and one of four different Argonaute (Argo) proteins (30). Once within the RISC, miRNAs bind to the 3'untranslated region (or UTR) of the target mRNA transcript. If the binding of the miRNA to the 3'UTR "seed sequence" exhibits perfect base pairing, the mRNA transcript is degraded and mRNA translation does not occur (31). If base pair binding homology between miRNA and 3' UTR of the mRNA is imperfect, mRNA translation is inhibited. While the majority of the literature supports the notion that miRNAs inhibit translation, there is some evidence that miRNAs can actually enhance translation through alterations in the Argo component of the RISC (23). Thus, while miRNAs appear to primarily regulate translation in an inhibitory fashion, they also may enhance translation in certain biological scenarios.

In addition to modulating protein expression within the same cell from which they are transcribed, miRNAs have also been proposed to elicit functions in distal cells/organs via transport through the circulation. Circulating miRNAs exist in cell free form and are thought to be protected from endogenous RNA activity by complexing with lipoproteins and/or residing in microvesicles, exosomes, and microparticles (Figure 1). Recipient cells may then take-up circulating miRNAs through endocytosis and/or by binding with cell surface receptors which recognize the miRNA-binding proteins to which the miRNAs are complexed. Once taken into the cell, miRNAs can elicit similar biological responses as miRNAs which are retained in the originating host cell. The observation that miRNAs are released by cells into the circulation has led to an intense effort in identifying disease-specific miRNAs which may be used as diagnostic markers for the disease. This has been evident in the study of many diseases with cancer being the most obvious. The simple premise that carcinoma cells release specific miRNAs into the circulation which can be used as diagnostic markers for the presence of these cancerous cells has led to intense investigation and great promise for miRNAs as potential diagnostic markers for disease. In the following paragraphs, we discuss our current knowledge on endometriotic lesion-derived miRNAs, their detection in serum and/or plasma and their potential promise as diagnostic markers for endometriosis.

MicroRNAs as diagnostic markers for disease

As described above, circulating miRNAs are resistant to RNase degradation and as such are stable within biological fluids. In addition, miRNAs are robustly expressed and conserved

among species. This latter characteristic bodes well for the use of animal model testing for miRNAs not only as diagnostic markers in experimental models but also offers a unique advantage in testing of treatments for disease and monitoring of disease status through circulating miRNA expression. These key characteristics, along with the possibility that circulating miRNAs may be expressed by specific disease tissue/cells, has led to an exhaustive amount of research examining their utility as diagnostic or prognostic markers for numerous diseases, of which, cancer has been the primary field of study.

Lawrie and colleagues were the first to demonstrate the presence of miRNAs in biological fluids when they reported that tumor associated miRNAs, miR155, miR210 and miR21, were detected in serum of patients with diffuse large B-cell lymphoma (32). This initial report led to intensive investigation of miRNAs as diagnostic/prognostic markers (over 600 publications to date) for many types of cancers including gastric (33), lung (34), and breast (35) among essentially all types of cancer. Beyond cancer, miRNAs have also been proposed as diagnostic markers for most diseases, including, but not limited to, cardiovascular disease (36), inflammatory bowel disease (37), Alzheimer's disease (38, 39), and other diseases of the central nervous system including Huntington's disease, multiple sclerosis, schizophrenia and bipolar disorder (39). The above information is provided to summarize the diversity among diseases for which miRNAs may be suitable non-invasive diagnostic markers. While it is beyond the scope of this review to discuss specific miRNAs which may be used as such markers and their cellular origin, we will focus, using a similar strategy, to discuss both endometriotic-specific miRNAs and serum miRNAs which may prove useful as potential non-invasive diagnostic markers for endometriosis.

MicroRNAs as diagnostic markers for endometriosis: Using circulating miRNA profiles as a diagnostic tool

As highlighted above, the use of miRNAs as serum diagnostic markers for other diseases has prompted a similar interest by the gynecologic community to begin to assess the utility of miRNAs as potential diagnostic markers for the presence of endometriosis. To date, three reports have been published examining circulating miRNAs as potential biomarkers for endometriosis, one assessing serum profiles (40) and the other assessing plasma profiles (41, 42). Differentially expressed miRNAs were first determined by miRNA array analysis and specific miRNAs were then further evaluated and identity confirmed by qRT-PCR analysis. For sake of brevity, we will limit discussion to differentially expressed miRNAs which were confirmed by qRT-PCR. For the full lists of miRNAs identified by array analysis, the reader is referred to the specific publication.

From the three current studies, several miRNAs have been identified as being differentially expressed in serum or plasma between women with and without endometriosis. Jia and colleagues (41) identified twenty-seven plasma miRNAs as being differentially expressed between women with and without endometriosis (greater than 2-fold difference). Of these, six miRNAs (miR-17-5p, miR-20a, miR-22, miR-15b-5p, miR-21 and miR-26a) were validated by qRT-PCR with only miR-17-5p, miR-20a and miR-22 being confirmed to be significantly lower in women with endometriosis. Interestingly, miR-17-5p and miR-20a were also identified as miRNAs which were differentially expressed in endometriotic

implant tissue compared to control tissue. Ramón and colleagues (43) reported significantly lower levels of miR-17-5p and miR-20a expression compared to eutopic endometrium from the same patient from which lesions were obtained, as well as compared to eutopic endometrium from non-endometriosis subjects (Table 2). miR-20a expression appeared to vary by lesion type, as in this same study, miR-20a expression in recto-vaginal lesions was significantly greater compared to control tissue (Table 1). Similarly, Zhao and coworkers (44) reported elevated miR-20a expression in ovarian endometrioma but this comparison was to ovarian, not endometrial tissue (Table 1).

Wang and colleagues (40) found 36 serum miRNAs to be down-regulated 10-fold or greater and 25 serum miRNAs to be up-regulated 10-fold or more in serum from women with endometriosis by array analysis. Of these, expression for five of the 61 miRNAs was further confirmed by qRT-PCR. miR-122 and miR-199a were demonstrated to be up-regulated and miR-145, miR-141*, miR-542-3p and miR-9* were down-regulated in serum from women with endometriosis. Of these miRNAs, miR-145 was reported to be over-expressed in endometriotic tissue in two independent studies (45, 46); a pattern of expression which suggests an inverse relationship between endometriotic implant miR-145 expression and serum levels of this miRNA. The remaining four serum miRNAs have not been identified as miRNAs whose expression significantly differs between endometriotic and control tissues.

Suryawanshi and colleagues (42) evaluated plasma miRNAs levels in women with endometriosis compared to women with endometriosis-associated ovarian cancer and controls. Twenty four candidate miRNAs were validated by qRT-PCR and the ten most differentially expressed miRNAs between groups were reported. Ten miRNAs, all of which were significantly higher in endometriosis subjects, were reported and included miR-16, miR-195, miR-191, miR-1974, miR-4284, miR-15b, miR-1978, miR-1979, miR-362-5p and miR-1973. However, of these, only miR1978 and miR-362-5p were not elevated in plasma from women with endometriosis-associated ovarian cancer compared to controls and neither of these miRNAs has been reported to be differentially expressed by endometriotic tissue.

Most recently, we have observed that endometriotic lesion miR-451 expression is elevated compared to matched eutopic controls (47; Table 1). We further noticed that this level of expression is associated with altered survival state of the lesion as those lesions with higher miR-451 expression co-expressed significantly higher levels of the apoptotic marker *PTEN* and significantly lower levels of proliferation marker, cyclin E1 (*CCNE1*). We are currently evaluating if these alterations in miR-451 expression are reflected in the serum of these same patients and the potential use of miR-451 as a diagnostic marker for the disease.

In summary, numerous miRNAs have been reported to be mis-expressed in either the serum or plasma of women with endometriosis. Of those miRNAs which have been reported to be elevated in the circulation of women with endometriosis, none of these have been demonstrated to be over-expressed by endometriotic tissue (43 – 50) raising the question on the mechanisms which lead to their elevated levels. Further, several studies have identified miRNAs which are under-expressed by endometriotic tissue (43, 45, 48, 51, 52; Table 2), but no difference in the level of expression of these miRNAs have been reported in serum or plasma between women with and without endometriosis.

MicroRNAs as diagnostic markers for endometriosis: The need for specificity and quantitative measurement

The study by Suryawanshi and colleagues (42) raises an important issue on specificity of miRNAs and endometriosis. For this discussion, we will focus on miR-199a and miR-122 as their expression has been reported to be elevated in the circulation of women with endometriosis but yet neither of these miRNAs has been reported to be significantly over-expressed by endometriotic lesions. Thus, the first question that may be raised is that if these miRNAs are not derived from the lesions themselves, what are the sources of these miRNAs and the mechanisms which generate their elevated levels?

Elevated circulating levels of both miR-199a and miR-122 have been reported for other, non-gynecologic diseases. Circulating levels of miR-199a are elevated in subjects with inflammatory bowel disease (IBD; 53), type 2 diabetes (54), and hepatocellular carcinoma (55) among other diseases. miR-122 is has been demonstrated to be elevated in the circulation of subjects with liver disease (56, 57), gastric cancer (58) and cardiovascular disease (59). While symptoms for these diseases differ from those of endometriosis and would undoubtedly assist in differential diagnosis, the goal for any diagnostic marker would be early detection, ideally before the manifestation of the more severe symptoms associated with the disease being diagnosed. Potential confounding factors could include the presence of inflammatory conditions which are associated with many diseases including IBD and cancer among others.

A second area which needs careful evaluation is the determination of the most optimal way to quantitate serum miRNAs as diagnostic markers and the potential need for an internal normalizing factor. The studies discussed in this review all report the levels of expression of the defined miRNAs as fold changes from controls. As these controls vary greatly from study to study, so does the reported fold change in miRNA expression. A more quantitative index will need to be established and this will require new technology as well as larger, more diverse sample sizes. Digital PCR (dPCR) is a technology that may allow for more precise quantitation of miRNAs concentrations in serum/plasma (60). Unlike qRT-PCR, dPCR is not dependent on the number of amplification cycles to determine the initial sample amount, thereby eliminating the reliance on uncertain exponential data to quantify target miRNAs (or nucleic acids in general) and therefore provides absolute quantification of the amount of target. dPCR coupled with larger sample sizes may allow for the development of normal ranges for given miRNAs in biological fluids from women without endometriosis. This “normal” range could then be used as a standard, much like blood glucose, cholesterol and the likes, to establish what are the normal and abnormal levels of a given miRNA to help distinguish or diagnosis those women with versus those without endometriosis.

miRNA values are often normalized to an endogenous “control” miRNA in biological samples to control for variation among samples in processing. There still remains great debate in how best to normalize miRNA values in tissue/cells and these as well as other concerns are also inherent when assessing circulating miRNA levels (61). Currently, U6 and miR-16 are the most common endogenous controls in the research of miRNAs in tissues and cells, both of which come under criticism as internal references for assessing biological fluid

miRNA levels as U6 lacks stability and levels of expression are influenced by sample processing and storage, while miR-16 levels have been shown to be altered in states of inflammation (40), stress (62) and in subjects with hepatocellular carcinoma (63). These results question the use of U6 and miR-16 as internal reference miRNAs suitable for normalization of test miRNA values.

An additional area which will need to be standardized is the processing and handling of biological fluid specimens. Differences in experimental approaches undoubtedly may introduce differences among study results. How blood samples are obtained and processed as well as how the RNA is extracted and amplified will need to be standardized. These not only hold true for potential limitation for miRNA quantitation but also the application of dPCR for these assessments.

Conclusions

In summary, endometriosis represents a major medical concern in women of reproductive age. One of the major hurdles in successful treatment of endometriosis that remains is the limitation in the process of timely disease diagnosis. MiRNAs represent potential biomarkers for early detection of the disease. miRNAs in general are more stable in circulation compared to most proteins and peptides; a characteristic that would reduce sample processing artifacts. Further, the potential for identification of endometriotic lesion-specific or enriched miRNAs as biomarkers would offer increased specificity and sensitivity compared to currently used or proposed biomarkers. While to date, endometriotic lesion-specific miRNAs have not been identified, the possibility still exists of identifying miRNAs (or a panel of miRNAs) whose expression may be altered in response to the presence of the disease. For this to occur and to achieve the goal of non-invasive diagnostic markers for the disease, larger studies must be conducted enrolling a diverse patient population to minimize overlap with other diseases and/or conditions. This will provide a range of normal miRNA values which can provide ranges to distinguish disease from non-disease. Further, assessment of circulating miRNA will require uniformity in specimen collection and processing to avoid the potential of false negatives or positives. Taking these factors into account will provide the best approach in identifying endometriotic lesion-specific or enriched miRNAs which may prove to be the ever-elusive diagnostic biomarker for this enigmatic disease.

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Figure 1.

Biogenesis, mechanism of action and extracellular secretion of microRNA (miRNA). Pri-miRNA are transcribed by polymerase II (POL II) in the nucleus and processed by Drosha into pre-miRNA. An alternative non-canonical pathway is generated by certain debranched introns, called 'mirtrons', which undergo splicing and mimic the structural features of pre-miRNA, entering the miRNA-processing pathway without Drosha-mediated cleavage. Exportin transports pre-miRNA molecules to the cytoplasm, where DICER generates miRNA-miRNA* duplexes. These are converted into single-strand mature miRNA and incorporated into the RNA-induced silencing complex (RISC), which sequence-specifically

binds to miRNA target sites on mRNA transcripts, effecting mRNA cleavage and degradation or, if the alignment is imperfect, repression of gene translation. Pre-miRNA are exported from cells via two mechanisms: (i) in multivesicular bodies (MVB), which release miRNA into the circulation via fusion with the cell membrane; and (ii) in association with RNA-binding proteins such as nucleophosmin 1 (NPM1), argonaute 2 (Ago2) or high-density lipoprotein (HDL). Circulating miRNA are taken up by the recipient cells either by endocytosis or, if protein bound, by receptor-mediated interactions at the cell surface. miRNA internalized by recipient cells can inhibit the expression of target protein-coding genes.

Table 1

miRNAs whose expression is higher in endometriotic lesions compared to control tissues.

miRNA	implant type ¹	control tissue comparison ²	Reference
miR-143 miR-145	OE	control and matched eutopic	46
miR-449a miR-34c miR-200a miR-200b miR-141	PL	adjacent peritoneum ³	49
miR-20a	OE	ovarian tissue	44
miR-29c	OE	control eutopic	50
miR-20a	RVL	control and matched eutopic	43
miR-21	OE, RVL		
miR-125a	OE, PL, RVL		
miR-222	OE, PL, RVL		
miR-202	OE	matched eutopic	48
miR-99a miR-126 miR-145	not specified	matched eutopic	45
miR-451	OE, PL, RVL	matched eutopic	47

¹ Implant type = OE = ovarian endometrioma, PL = peritoneal lesion, RVL = recto-vaginal lesion

² Control tissue =

miRNAs in **bold print** represent those reported to be differentially expressed in multiple reports.

Table 2

miRNAs whose expression is lower in endometriotic lesions compared to control tissues.

miRNA	implant type ¹	control tissue comparison ²	Reference
miR-202-3p miR-424-5p miR-449-3p miR-556-3p	OE, PL	control and matched eutopic	52
miR-126	OE	control and matched eutopic	51
miR-15 miR-17-5p miR-20a	OE, PL	control and matched eutopic	43
miR-200a miR-200b miR-200c, miR-182	OE	matched eutopic	48
miR-141 miR-200b miR-424	not specified	matched eutopic	45

¹ Implant type = OE = ovarian endometrioma, PL = peritoneal lesion, RVL = recto-vaginal lesion

² Control tissue =

miRNAs in **bold print** represent those reported to be differentially expressed in multiple reports.