Published in final edited form as:
Gynecol Oncol. 2010 September ; 118(3): 251–257. doi:10.1016/j.ygyno.2010.05.010.

MicroRNA Signatures Differentiate Uterine Cancer Tumor Subtypes

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Abstract

Purpose—Endometrial cancer (EC) is the most common gynecologic malignancy. Type I EC has a favorable prognosis, while type II ECs account for half of all treatment failures. Little knowledge of the biological differences is available to predict EC outcomes besides their pathological distinctions. MicroRNAs (miRNA) are a family of non-translated RNAs important in regulating oncogenic pathways. Mis-expression patterns of miRNAs in EC, as well as differences in miRNA expression patterns between the subtypes of EC has not been previously evaluated. Our purpose was to identify miRNA profiles of EC subtypes, and to identify miRNAs associated with these subtypes to ultimately understand the different biological behavior between these subtypes.

Methods—95 fresh/frozen and paraffin embedded samples of endometrial type I and II cancer, carcinosarcomas and benign endometrial samples were collected. MiRNA expression profiles were evaluated by microarray analysis. Statistical analysis was performed.

Results—Distinct miRNA signatures in tumor versus normal samples and in endometrioid vs. uterine papillary serous carcinomas exist. Additionally, carcinosarcomas have a unique miRNA signature from either the type I or II epithelial tumors.

Conclusions—We hypothesize that further understanding the miRNAs that separate these subtypes of EC will lead to biological insights into the different behavior of these tumors.

Keywords

microRNAs; endometrial cancer; carcinosarcoma; uterine papillary serous carcinoma

Introduction

Cancer of the uterine corpus is the most common gynecologic malignancy and the fourth most common cancer in women. (1) The American Cancer Society estimates that 40,100

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Conflict of Interest Statement: The authors declare that there are no conflicts of interest.

women will be diagnosed with endometrial cancer in the United States in 2009 – and 7,470 of these women will die from their disease. (2) However, endometrial carcinoma is a varied disease with five-year survival rates for localized, regional, and metastatic disease reported to be 95, 67, and 23 percent, respectively. (3) The disparity in overall patient survival is clarified by classification of endometrial carcinomas into two types of tumors carrying distinctly different characterization and prognosis. (4) Type I cancers, which are estrogen related, occur mainly in perimenopausal and obese patients, are usually low stage and low grade (frequently occurring in the background of hyperplasia) and have an excellent prognosis. (4) Type II endometrial carcinomas tend to spread aggressively and have a poor prognosis. They are unrelated to estrogen stimulation and occur in older non-obese women. Women with type II endometrial cancer have adverse histologic features, including poorly differentiated Grade 3 tumors, papillary serous and clear cell tumors. The mean age of Type II tumors is 68 years and the overall 5-year survival is only 46%. (4) Uterine papillary serous carcinomas carry a particularly poor prognosis, with extrauterine spread found in up to 72% of patients at diagnosis.

Carcinomas account for 95% of uterine malignancies and arise from the epithelial layer of the uterus. The prevalence of pathological subtype of this tissue is reported to be: adenocarcinoma as 89 percent, uterine papillary serous carcinomas as 6 percent and clear cell tumors as 5 percent. (9) (10) The remaining 6 percent of uterine cancers are sarcomas (consisting of leiomyosarcomas and endometrial stromal sarcomas) and carcinosarcomas. Carcinosarcomas have historically been classified as sarcomas, however, recent nomenclature categorizes these tumors as carcinomas. Carcinosarcomas carry a very poor prognosis with the five-year survival of 25 to 35 percent. (11) In these cancers malignant epithelial and stromal components contribute to the architecture of the tumor. The carcinomatous element is usually grade 3 endometrioid, clear cell or papillary serous histology. Many investigators have attempted to determine if these tumors represent collision tumors (made of 2 genetically distinct cell populations) or combination tumors (both cell types arise from a common progenitor cell that is capable of multlineage differentiation). (12) Immunohistochemical studies support the later, that precursor (stem) cells give rise to both components during the histogenesis of the tumor. (13) Data confirms that the carcinomatous element is the predominant element and that the sarcomatous component is derived from the metaplasia or from a stem cell that undergoes divergent differentiation. (14) Based on these findings, uterine carcinosarcomas are now classified as a type of non-endometrioid endometrial cancer rather than a uterine sarcoma by most recent treatment guidelines from the National Comprehensive Cancer Network. However, treatment of these tumors is still debated, with some endorsing chemotherapy appropriate for the high-grade epithelial component while others advocating sarcoma based adjuvant treatment. (15)

Varying risk factors and prognosis between the different subtypes of uterine cancer suggest that they harbor distinct molecular alterations, some of which have been previously delineated through single gene analysis. Mutations of the p53 gene have been found in up to 90% of epithelial tumors that are grade 3 or papillary serous carcinoma but are absent in grade one type I tumors. (16) The presence of p53 overexpression and high S phase fraction increases the risk of recurrence seven-fold, and the risk of cancer-related death almost 10-fold when compared to tumors with neither factor. (17) In a multivariate analysis p53 was identified as the strongest predictor of survival. (18) In contrast, PTEN, a tumor suppressor gene on chromosome 10, is often mutated or deleted and is associated with endometrioid histology and a favorable prognosis. (19) Other altered oncogene/tumor suppressor gene expression patterns have been demonstrated in endometrial cancer; MDR-1 and ER/PR positivity have been reported to be favorable prognostic factors, while microsatellite instability, HER2/neu receptor positivity, Ki 67, PCNA and EGF-R over-expression have

been shown to carry an unfavorable prognosis. (20-25) Expression of the Her-2/neu gene has been shown to be present in 27 percent of women with metastatic disease compared to 4 percent of patients where disease is limited to the uterus. (26)

Although the above findings reflect important molecular insights into uterine cancer, a better and more global understanding is necessary to both identify new targets for therapy and to better predict an individual's outcome. MicroRNAs (miRNAs) are a class of 22-nucleotide noncoding RNAs, which are evolutionarily conserved and function by negatively regulating gene expression at the post-transcriptional level. MiRNAs are global regulatory RNAs that each control hundreds of mRNA transcripts. Recent studies have shown that miRNAs are aberrantly expressed in virtually all human cancer types (27) and that specific miRNAs misregulated in each cancer type may act as biomarkers of outcome for that cancer type. (28) The miRNA signatures of uterine cancer or specifically uterine cancer subtypes has not been previously explored, prompting the current investigation.

By miRNA microarray we were able to identify unique miRNA signatures that could separate type I (endometrioid) from type II (papillary serous) uterine cancers. Furthermore, we found that carcinosarcomas have a distinct miRNA signature that is unique from epithelial uterine cancer miRNA signatures, adding further credence to the belief that they are biologically unique tumors.

Materials and Methods

Fresh/Frozen Tissue Collection

After approval from the Human Investigation Committee at Yale University, uterine tumor samples and normal endometrial tissues were obtained from untreated patients undergoing surgery at Yale-New Haven Hospital (New Haven, CT). All patients underwent staging surgery as initial treatment. Patient data was collected including age, race, parity and risk factors. All tumors were from primary sites. The carcinoma samples were histologically examined for the presence of tumor. Specimens were immediately snap-frozen and stored at -80 C. The fresh/frozen tissue collection used for microarray analysis included five benign endometrial tissues, eleven endometrioid adenocarcinomas, six papillary serous tumors and six carcinosarcomas. All were examined microscopically and microdissected to ensure greater then 75% tumor cellularity.

Paraffin Embedded Uterine Tumors

For addition tumor specimens paraffin embedded tumors (FFPE) were microdissected and used for microarray analysis. In all cases sections of tumor used had greater then 75% tumor cellularity. Twenty-one papillary serous tumors from Yale were identified, microdissected, analyzed by microarray and included in the analysis. Forty-six endometrioid adenocarcinomas from RTOG (Radiation Therapy Oncology Group) trials 9708 and 9905 were microdissected, analyzed by microarray and used in the analysis. There was no difference in miRNA signatures identified between fresh/frozen and FFPE tissues in these analyses (data not shown).

RNA Extraction

Total RNA isolation, including small RNAs, was performed with the mirVana RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions for all fresh frozen tissue. Each sample was derived from a single specimen. Integrity of the RNA was assessed using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). RNA was extracted from paraffin-embedded slides using Trizol, per protocol.

MiRNA Profiling

cDNA was synthesized from 160ng-800 ng of total RNA using TaqMan MiRNA primers and the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems). Expression of 384 mature miRNAs was then analyzed with the Asuragen TLDA assay and the Applied Biosystems 7900 Taqman Real-Time PCR machine in accordance with manufacturer's instructions. Fold changes in miRNA expression between benign and malignant samples as well as between different malignant subtypes were determined by delta-delta CT values. Normalization was done to two internal small RNA controls RNU44 and RNU48. In the majority of samples 102 miRNAs were detected from the 384 measured, and a CT cutoff of 34 was used in all of the samples. To confirm data the first 12 samples were run in duplicate, and all were statistically similar in results.

Statistical Analysis

All normalization and data analyses were performed in the statistical programming environment R (29) using customized functions and functions available from Bioconductor (30) and the limma software package. We normalized the sample input CT values for each miRNA by quantitating small nuclear RNAs using the TaqMan(R) MiRNA Assay Controls (Applied Biosystems). Each of the 8 pools are normalized separately by the associated small nuclear RNAs. The intensities are scaled to have similar distributions across the entire series of samples to have the same median absolute deviation across samples. The miRNA expression data for different tumor types was analyzed together by using linear modeling methods. (31) The linear models allowed for general changes in gene expression between different conditions and across different biological replicates. Assessment of differential expression was assessed using a moderated t-statistic. P values were adjusted for multiple testing based on all the miRNAs which were expressed in samples (excluding control and unexpressed miRNAs) according to the method of Benjamini and Hochberg (32) to control the false discovery rate. Hierarchical clustering was performed with Pearson correlation and average linkage, based on miRNAs selected for differential expression between any of the groups of interest.

Patient Characteristics

Table 1 describes the clinicopathologic parameters of the study population. Pathologic examination confirmed malignancies in 90 patients while 5 patients had no malignancy and represent the benign cases.

Results

A miRNA expression signature discriminates type I endometrial cancer from benign endometrium

When the expression of miRNAs was compared between endometrioid endometrial cancer samples and normal endometrial benign tissues, 10 of the 384 miRNAs showed significantly differential expression. Several miRNAs were significantly up-regulated (with FDR < 0.03) in endometrial carcinoma samples, while two miRNAs were down-regulated (Table 2, Supplemental Figure 1). Among the top differentially expressed miRNAs, *miR-205*, *miR-182* and *miR-200a* are most up regulated in endometrioid samples while *mir-411* was most down-regulated in cancerous samples compared to benign (Table 2). There was no significant difference between Grade 1 and 3 endometrioid tumors in this analysis, so they were grouped together.

A miRNA expression signature discriminates between Type I (endometrioid) from Type II (uterine papillary serous) cancers

We next compared miRNA expression patterns between endometrioid and papillary serous tumors (UPSC). MiRNA expression patterns were also distinct between these carcinomas (Supplemental Figure 2). Eight miRNAs were significantly lower in endometrioid tumors compared to UPSC tumors (with FDR < 0.025) (Table 3). The most down-regulated miRNAs in endometrioid tumors compared to UPSC included *miR-19a* and *miR-19b*.

A miRNA expression signature discriminates between uterine carcinomas and uterine carcinosarcomas

We compared miRNA signatures between carcinosarcomas and the epithelial type uterine tumors. We found that carcinosarcomas have a unique miRNA signature that is unlike either endometrioid (Figure 1A) or uterine papillary serous tumors (Figure 1B). Some unique miRNAs differentiate carcinosarcomas from type I endometrioid tumors and others from UPSC tumors. In endometrioid tumors compared to carcinosarcomas specifically, *miR-133a* is upregulated in endometrioid tumors, while *miR-19a and miR-19b* are down-regulated (Table 4). When comparing UPSC to carcinosarcomas, *miR-22* is upregulated in UPSCs while *miR-182* is down-regulated with a FDR < 0.05. (Table 5). Interestingly, there were also miRNAs that were similarly misregulated in endometrioid and UPSC tumors compared to carcinosarcomas: *miR-518b* was upregulated and *miR-301, miR-20b* and *miR-487b* were down-regulated. These miRNAs may have a specific role in carcinosarcomas compared to carcinomas of the uterus, and may warrant further investigation.

MiRNA signatures slightly differ by age and ethnicity

MiRNA profiles were compared between patients of different ages and ethnicities, including Caucasian and AA, to determine if miRNA expression patterns would vary depending on these factors. We found only one miRNA, *miR-486*, that was significantly higher in younger patients with endometrioid uterine cancer ($p<0.03$, Supplemental Figure 3). This was primarily driven by elderly AA EAC patients where the expression was virtually absent. While these results are based on small sample sized they suggest that ethnicity and age should be considered in miRNA signatures.

Discussion

We report unique miRNA signatures for endometrial type I endometrioid carcinomas, type II papillary serous carcinomas and uterine carcinosarcomas. While multiple human cancer miRNA signatures have been described, only breast cancer has been previously profiled by subtype. (33-38) Perhaps because our numbers were small, there was no significant miRNA subset classifying different stages of disease, and only one that could separate patients by age, *miR-486*. However, our findings support the unique biology of these tumor types, and may represent future means to distinguish these tumors in difficult cases as well as to identify novel targets for therapy.

We have demonstrated both up-regulation and down-regulation of miRNAs in uterine endometrioid cancer compared to benign specimens. There were several miRNAs of interest that were different between benign endometrium and endometrioid cancers. Up regulation of the *mir-200* family has been recently demonstrated in well-differentiated cancers, and is seen in our endometrioid samples. (39) Likewise, expression of *miR-183* is inversely correlated with the metastatic potential of lung cancer cells. A 2–3 fold decrease of *miR-183* was demonstrated in highly metastatic lung cancer cells versus non-metastatic counterparts derived from same parental cell lines. (40) Finding that *mir-183* is relatively high in endometrioid cancer, which metastasizes infrequently, is thus not surprising. We find

miR-205 and *miR-182* to be up-regulated in endometrioid carcinomas. *MiR-205* has previously been described to be up-regulated in ovarian cancer as well as bladder and kidney cancers. (41-42) *MiR-205* is down-regulated in prostate cancer and esophageal cancer compared to normal tissue. (43-44) *MiR-205* along with the *mir-200* family has been demonstrated to cooperatively regulate expression of the E-cadherin transcriptional repressors ZEB1 (also known as δEF1) and SIP1 (also known as ZEB2), factors previously implicated in epithelial to mesenchymal transition and tumor metastasis. (45) *MiR-182*, member of a miRNA cluster in a chromosomal locus (7q31–34), up-regulated in endometrioid cancer is also up-regulated in melanoma cell lines and tumor samples. *MiR-182* expression stimulates migration of melanoma cells in vitro and their metastatic potential in vivo, whereas *miR*-182 down-regulation inhibits tumor invasion and triggers apoptosis. (46)

Compared to the endometrioid subtypes (Type I), UPSC (Type II) had unique miRNA signatures, and showed higher miRNA expression of some specific miRNAs. *MiR-19a & b*, the key oncogenic component of *mir-17-92*, is up-regulated in UPSC tumors. These miRNAs have been shown to be altered in hematologic cancers and to promote lymphomagenesis in vivo. (47) The oncogenic activity of *miR-19* is has been shown to be at least partly due to its repression of the tumor suppressor *Pten*. (48) *MiR*-101, another *miRNA* altered in UPSC, is down-regulated in hepatocellular carcinoma and was further reported to promote apoptosis and affect tumorigenicity. (49) *MiR*-30e-5p is also up-regulated in UPSC tumors. Interestingly, this *miRNA* has been reported to be aberrant in ovarian and peritoneal endometriosis. Its up-regulation was described to be specific to endometriosis independently from the site of the lesion. (50) Furthermore, up-regulation of *miR*-452 seen in UPSC, has been shown to be associated with lymph node positivity and serve as a prognostic marker for death in urothelial cancers. (51) This described finding in consistent with UPSC tumors having overall poor prognosis and widespread metastasis to the lymph nodes. *MiR*-29c, also misregulated in UPSC, is up-regulated in epithelial mesotheliomas. Increased expression of hsa-miR-29c predicted a more favorable prognosis in these tumors, and it's overexpression of resulted in significantly decreased proliferation, migration, invasion, and colony formation in these tumor cell lines. (52)

We have further shown that uterine carcinosarcomas demonstrate a unique miRNA signature, easily distinguishing these tumors from endometrial epithelial cancers. This is interesting as carcinosarcomas consist of both epithelial and sarcomatous components, and many advocate treating the epithelial component. However, our studies suggest that based on the miRNA signature of these tumors, they are biologically unique, and further support the hypothesis that these tumors likely require therapy unique from other epithelial tumors. Certain *miRNAs* appear to be consistently altered in the carcinosarcomas compared to epithelial tumors. *MiR*-518b is down-regulated in carcinosarcomas compared to both endometrioid and UPSC tumors, while *miR*-20b, *miR*-301 and *miR*-487 are up-regulated. *MiR*-20b has been described to accumulate in tumor cells and to play an oncogenic role. (53) *MiR*-20b is a regulator of VEGF, the critical angiogenic factor in response to hypoxia. (54) Low expression of *miR-20b* inhibits tumor cell growth but gives tumor cell more resistance to apoptosis in hypoxia. (55)

While limitations of our study were the lack of clinical follow up for these patients to correlate miRNA signatures with outcome, this is the first report in our knowledge of different miRNA signatures across these subtypes of uterine cancer. Due to the large number of patient samples the differences in our miRNA signatures are strongly significant and represent real differences between these tumor subtypes. Because these subtypes have such different biological behavior, their baseline differences in miRNA signatures are certainly likely to represent meaningful insight into their behavior. Our findings thus represent insight

into the basic biological differences between these types of uterine cancers, and when further validated may represent the first steps towards identifying important biomarkers of patient outcome and targets for therapy for these patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the RTOG Translational Research Program, funded through grant U10CA21661 by the National Cancer Institute. This research was further supported in part by the Yale Center of Excellence in Molecular Hematology, NIH P30 DK072442. JW was supported by K08 (CA124484).

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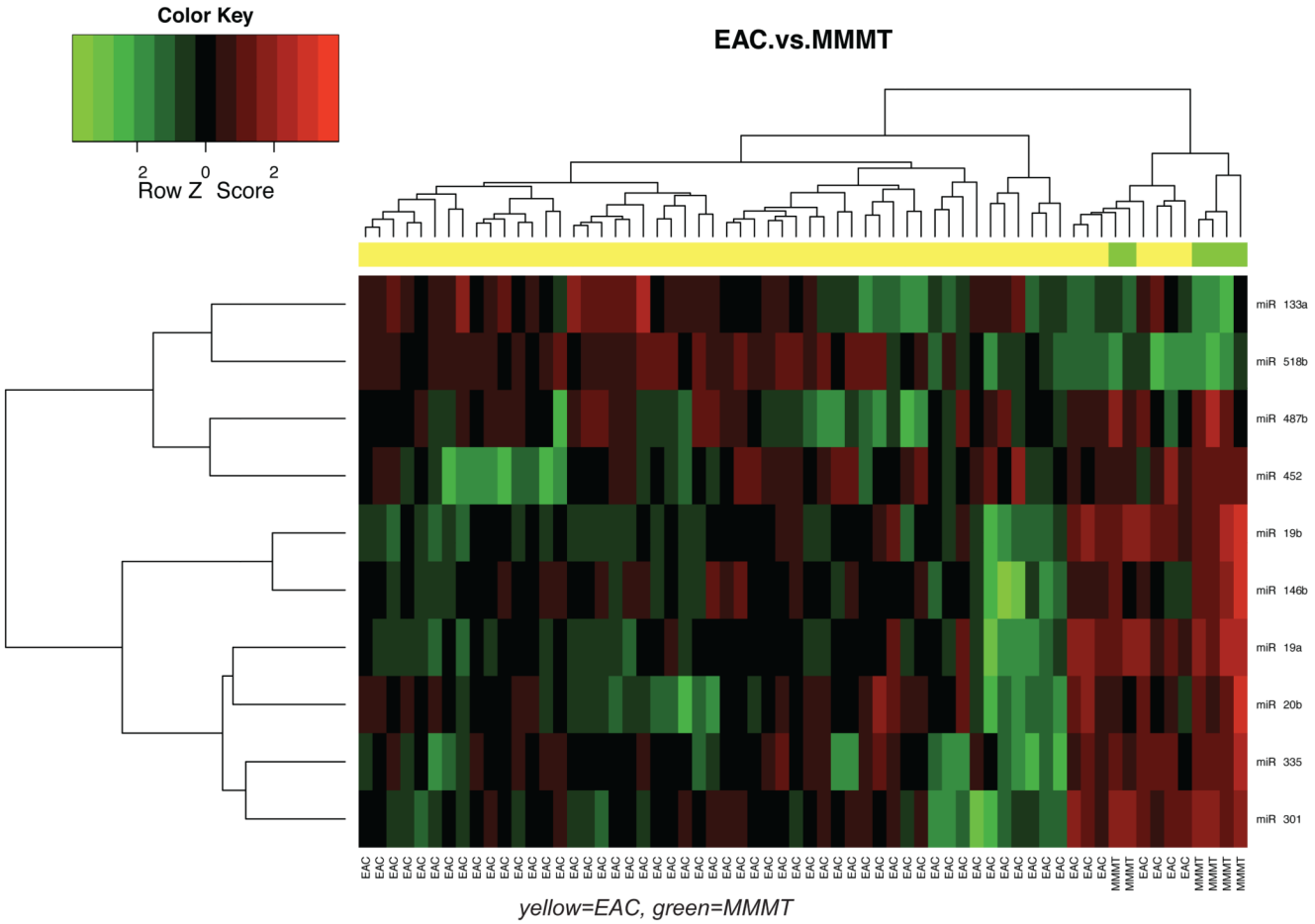

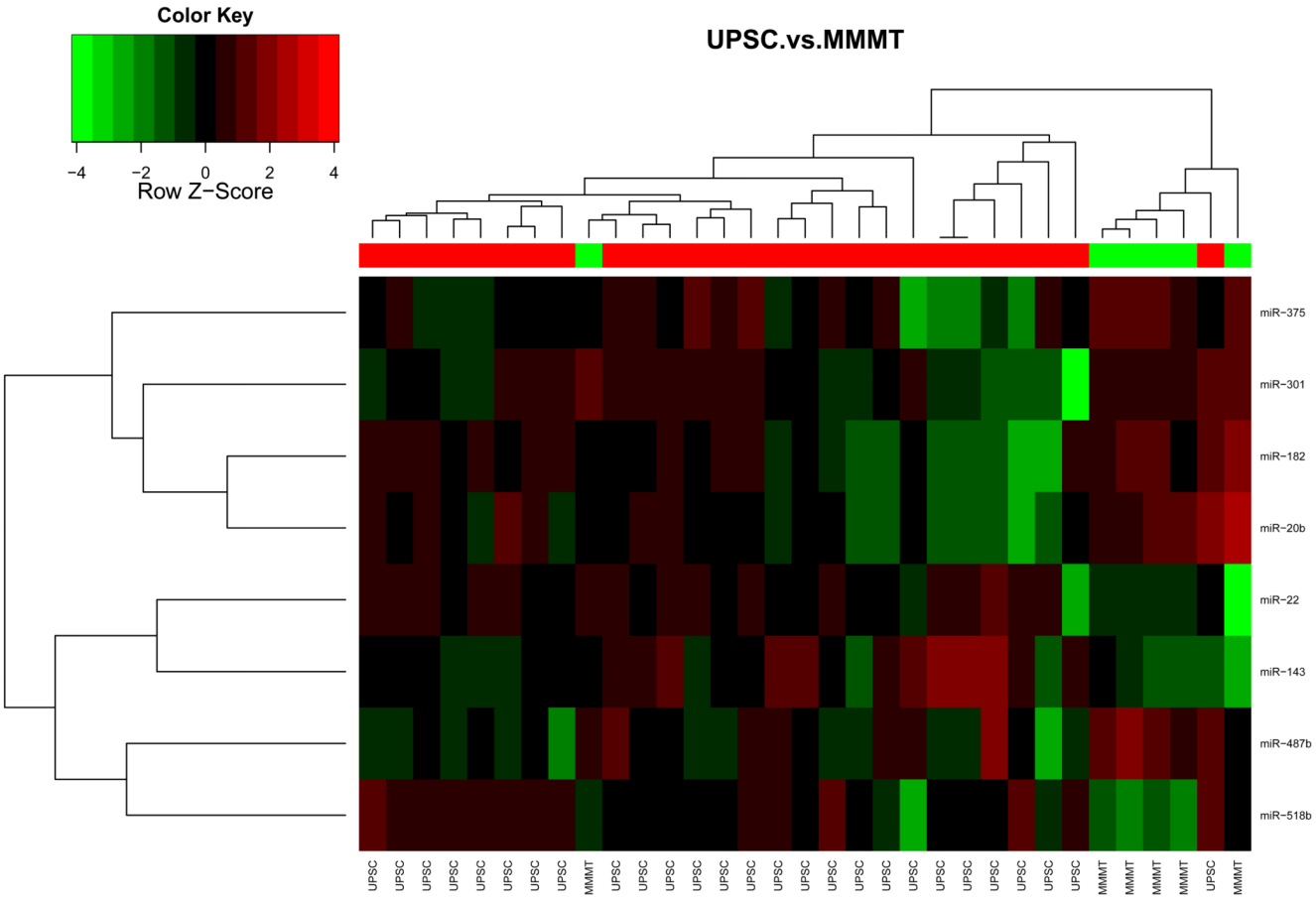

Figure 1. Comparison of miRNA expression patterns in endometrioid carcinoma, UPSC and carcinosarcoma

A. A heat map of 6 Carcinosarcomas and 57 endometrioid endometrial carcinoma samples indicates that there is a difference in miRNA expression between the two groups. **B.** Comparison of 6 Carcinosarcomas and 27 UPSC show that there is a difference in miRNA expression between these groups. MicroRNA expression is displayed as higher (red) or lower (green).

Table 1

Patient characteristics

Clinicopathologic Parameters (n=95)	
Pathology:	
Malignant	90
Endometrioid	57
Uterine Papillary Serous Carcinoma	27
Carcinosarcoma (MMMT)	6
Benign	5
Age:	
Malignant	
Endometrioid	60 (36-82)
Uterine Papillary Serous Carcinoma	70 (55-90)
Carcinosarcoma (MMMT)	62 (48-75)
Benign	53 (45-63)
Ethnicity:	
Malignant	
Endometrioid	
Caucasian	46
African American	8
Hispanic	3
Uterine Papillary Serous Carcinoma	
Caucasian	18
African American	8
Unknown	1
Carcinosarcoma (MMMT)	6
Caucasian	5
African American	1
Benign	
Caucasian	2
African American	1
Hispanic	2
FIGO stage:	
Endometrioid carcinoma	
Stage I	27
Stage II	12
Stage III	18
Uterine Papillary Serous carcinoma	
Stage I	8
Stage II	5
Stage III	7
Stage IV	7

Carcinosarcomas	
Stage I	4
Stage III	2

Table 2

Type 1 endometrioid uterine carcinoma miRNA signatures compared to benign endometrium miRNA signatures

Upregulated	Fold Change	Nominal P Value	Adjusted P. Value (FDR)
miR-650	4.8	6.3E-05	0.0065
miR-183	5.3	1.2E-04	0.0065
miR-572	4.5	1.5E-04	0.0065
miR-200a	5.4	1.7E-04	0.0065
miR-182	6.2	4.7E-04	0.0111
miR-622	4.8	5.0E-04	0.0111
miR-34a	3.7	1.7E-03	0.0301
miR-205	6.7	1.9E-03	0.0301
Downregulated			
miR-411	-3.8	4.8E-03	0.0111
miR-487b	-2.7	1.9E-03	0.0301

Table 3

Endometrioid carcinoma miRNA signatures compared to UPSC carcinoma miRNA signatures

Downregulated	Fold Change	Nominal P Value	Adjusted P. Value (FDR)
miR-19a	-5.1	7.6E-08	1.2E-05
miR-19b	-4.2	2.0E-06	1.5E-04
miR-30e-5p	-3.8	7.2E-06	3.7E-04
miR-101	-3.8	1.6E-05	6.1E-04
miR-452	-3.9	2.5E-04	6.5E-03
miR-15a	-3.3	7.2E-04	1.3E-02
miR-29c	-3.7	1.1E-03	1.6E-02
miR-382	-3.8	1.2E-03	1.6E-02

Table 4
Endometrioid miRNA signatures compared to carcinosarcoma miRNA signatures

Upregulated	Fold Change	Nominal P Value	Adjusted P. Value (FDR)
miR-518b	2.4	2.6E-05	1.4E-03
miR-133a	2.5	3.6E-04	8.3E-03
Downregulated			
miR-19a	-7.0	1.4E-07	2.2E-05
miR-19b	-5.7	2.8E-06	2.2E-04
miR-301	-6.2	4.9E-05	1.9E-03
miR-146b	-4.6	1.4E-04	4.3E-03
miR-335	-5.1	3.8E-04	8.3E-03
miR-487b	-4.8	8.0E-04	1.6E-02
miR-20b	-5.2	1.2E-03	2.0E-02
miR-452	-4.8	2.7E-03	4.0E-02

Table 5
UPSC miRNA signatures compared to carcinosarcoma miRNA signatures

Upregulated	Fold Change	Nominal P Value	Adjusted P. Value (FDR)
miR-22	2.4	2.6E-05	1.4E-03
miR-518b	2.5	3.6E-04	8.3E-03
miR-143			
Downregulated			
miR-182	-7.0	1.4E-07	2.2E-05
miR-301	-6.2	4.9E-05	1.9E-03
miR-20b	-4.6	1.4E-04	4.3E-03
miR-375	-5.1	3.8E-04	8.3E-03
miR-487b	-4.8	8.0E-04	1.6E-02