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MicroRNA signatures differentiate uterine cancer tumor subtypes

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ABSTRACT

Objective. 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. Ninety-five 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.

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 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 5-year survival rates for localized, regional, and metastatic disease reported to be 95%, 67%, and 23%, 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 peri-menopausal 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 [5-8].

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%, uterine papillary serous carcinomas as 6% and clear cell tumors as 5% [9,10]. The remaining 6% 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 5-year survival of 25 to 35% [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 multilineage differentiation) [12]. Immunohistochemical studies

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support the latter, 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 7-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 overexpression 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% of women with metastatic disease compared to 4% 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 have 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 than 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 than 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 160–800 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