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The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform

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Abstract

Objective. To determine the utility of serum miRNAs as biomarkers for epithelial ovarian cancer.

Methods. Twenty-eight patients with histologically confirmed epithelial ovarian cancer were identified from a tissue and serum bank. Serum was collected prior to definitive therapy. Fifteen unmatched, healthy controls were used for comparison. Serum was obtained from all patients. RNA was extracted using a derivation of the single step Trizol method. The RNA from 9 cancer specimens was compared to 4 normal specimens with real-time PCR using the TaqMan Array Human MicroRNA panel. Twenty-one miRNAs were differentially expressed between normal and patient serum. Real-time PCR for the 21 individual miRNAs was performed on the remaining 19 cancer specimens and 11 normal specimens.

Results. Eight miRNAs of the original twenty-one were identified that were significantly differentially expressed between cancer and normal specimens using the comparative C_t method. MiRNAs-21, 92, 93, 126 and 29a were significantly over-expressed in the serum from cancer patients compared to controls ($p<.01$). MiRNAs-155, 127 and 99b were significantly under-expressed ($p<.01$). Additionally, miRs-21, 92 and 93 were over-expressed in 3 patients with normal pre-operative CA-125.

Conclusion. We demonstrate that the extraction of RNA and subsequent identification of miRNAs from the serum of individuals diagnosed with ovarian cancer is feasible. Real-time PCR-based microarray is a novel and practical means to performing high-throughput investigation of serum RNA samples. miRNAs-21, 92 and 93 are known oncogenes with therapeutic and biomarker potential.

Keywords: Ovarian cancer; MicroRNA; Biomarkers

Introduction

In 2008, it is expected that 20,180 women will be diagnosed with ovarian cancer and 15,310 will succumb to the disease [1]. Ovarian cancer is a devastating illness in which only 20% of patients are diagnosed with stage I disease [2]. The poor prognosis associated with ovarian cancer is multi-factorial; a lack of minimally invasive, early detection tests, subtle symptom development and tumor chemo-resistance. Even with the advent of chemo-resistance assays it is still difficult to predict drug resistance and only 10–15% of patients will remain in prolonged remission after initial cytotoxic therapy.

While annual pelvic examination is widely practiced, it lacks the sensitivity to be used a screening strategy for ovarian cancer [3]. Women at high risk for ovarian cancer may typically undergo screening with trans-vaginal ultrasound and serum CA-125. CA-125, however, remains a poor marker for early stage disease with a documented sensitivity of 40% [4,5]. Additionally, it has been shown that even in a high-risk, screened population, incident cases are still more likely to be advanced stage [6]. The identification of biomarkers that may assist in treatment planning and prediction of chemotherapy outcomes is highly desirable in this population of patients.

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There is emerging research about the role of microRNAs in a variety of pathologic conditions; including both solid and hematologic malignancies. MicroRNAs (miRNAs) are small, 22–25 nucleotide non-coding sequences of RNA. These sequences control gene expression either by translational repression or degradation of the messenger RNA transcript after targeting the 3′UTR. Early studies with *Caenorhabditis elegans* showed that a great number of these sequences are highly conserved across all species, demonstrating the important roles that miRNAs play in cellular differentiation, proliferation and cell cycle control [7]. It is now recognized that miRNAs are frequently de-regulated in malignancy. Under-expressed miRNAs such as let-7 in lung cancer and mirs-15/16 in leukemia, are tumor suppressor genes, suppressing Ras and BCL2 respectively [8,9]. Over-expressed miRNAs such as mir-21 and the cluster mir-17–92, are oncogenes (oncomirs), targeting tumor suppressors PTEN and E2F1 in solid and hematologic malignancies respectively [10,11]. While miRNA research in gynecologic malignancies is in its infancy, the miRNA signature profile of ovarian cancer has recently been published [12–14].

The diagnostic and prognostic utility of circulating RNAs in both benign and malignant conditions has recently been revealed. Placental-associated circulating miRNAs correlate with pregnancy progression [15]. In malignant states, circulating mRNAs in renal cell carcinoma patients [16] as well as miRNAs from the serum of patients with diffuse large B cell lymphoma [17] have been shown to be stable and highly predictive of malignancy as well as survival. Recently, it has been demonstrated that the miRNA signature of circulating tumor exosomes of ovarian cancer patients demonstrates high correlation with miRNA expression of the primary tumor [18]. Ovarian cancer remains a disease for which improved non-invasive, serum screening tests are highly desirable. We offer a description of miRNA extraction from the serum of ovarian cancer patients, the differential expression of a number of these miRNAs between patients and healthy controls as well as a novel real-time PCR microarray detection method.

Methods

Following approval from the Institutional Review Board of The Ohio State University College of Medicine we analyzed serum samples from 28 patients with newly diagnosed ovarian cancer and 15 normal controls. These serum samples were collected at the time of initial consultation, prior to definitive surgical management and/or adjuvant therapy. The serum was obtained as part of a prospective tissue and serum procurement study and was stored at −80 °C. Fresh serum was obtained from 15 healthy women who volunteered to serve as controls. The frozen serum was thawed and RNA was extracted from the patient and control populations simultaneously. None of the healthy controls had previously been diagnosed with a malignancy.

RNA was extracted from 250 μl of serum using the Tri-Reagent BD (Molecular Research Center, Inc., Cincinnati, OH) as described by the manufacturer. RNA quality was assessed with the ThermoScientific NanoDrop1000 (Thermo Fisher Scientific, Inc., Waltham, MA). MicroRNA expression profiling was performed with RNA from 4 controls and 9 cancer patients utilizing the TaqMan Array Human MicroRNA Panel (v.1, Applied Biosystems, Foster City, CA) using 50 ng of RNA per port for a total of 400 ng. This array contains 365 miRNA targets as well as endogenous controls. Normalization was performed with the small nuclear RNAs (snRNAs) U44 and U48. These snRNAs are stably expressed reference genes suitable for use as normalizers in TaqMan assays.

In addition to identifying differentially expressed miRNAs on the microarray panel, a second goal was to identify miRNAs that may serve as normalizers given the lack of published data on the subject. Twenty-one miRNAs from the expression profile were empirically chosen for further examination in control and patient serum (11 controls and 19 patients). These were chosen based on apparent C_t differences of 4 cycles or greater between controls and patients. Two miRNAs (142-3p and 16) were identified as potential normalizers given consistent expression across all patient and control samples. For the miRNAs of interest the single tube TaqMan MicroRNA Assays were used. All reagents, primers and probes were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA). One nanogram of RNA per sample was used for the assays. MiRNA-142-3p was used as a normalizer. All RT reactions, including no-template (no cDNA) controls and minus controls (no reverse transcriptase), were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Comparative real-time PCR was performed in triplicate, including no-template controls.

Expression of the microRNAs was calculated utilizing the comparative C_t. method. Statistical analysis was performed with STATA v. 10 (College Station, TX). Expression was compared using the Mann–Whitney test. P-values < 0.05 were considered statistically significant.

Results

Twenty-eight patients with epithelial ovarian cancer were included in this study. Stage breakdown was as follows: stage I-8 (28.5%), stage II-2 (7.1%), stage III-8 (28.5%) stage IV-10 (35.7%). Histologic breakdown was as follows: serous (60%), clear cell (21.2%), endometrioid (12%), mucinous (6%). Median age was 57 years (age range 34–79). Similar to most groups with ovarian cancer, the majority (66%) had stage III or IV disease, and was predominately (60%) serous histology.

Primary miRNA expression profiling with microarray identified 23 miRNAs (including 2 normalizers) of interest. We created a Venn diagram in order to compare our 23 miRNAs of interest from our initial test set with known miRNA signature profiles. There were 10 miRNAs of interest in our group that were in common with miRNAs that have been published in the literature as part of the miRNA signatures of ovarian cancer (Fig. 1).

On follow up quantitative RT-PCR of the 21 miRNAs, 5 miRNAs were over-expressed (miRNAs-21, 29a, 92, 93 and

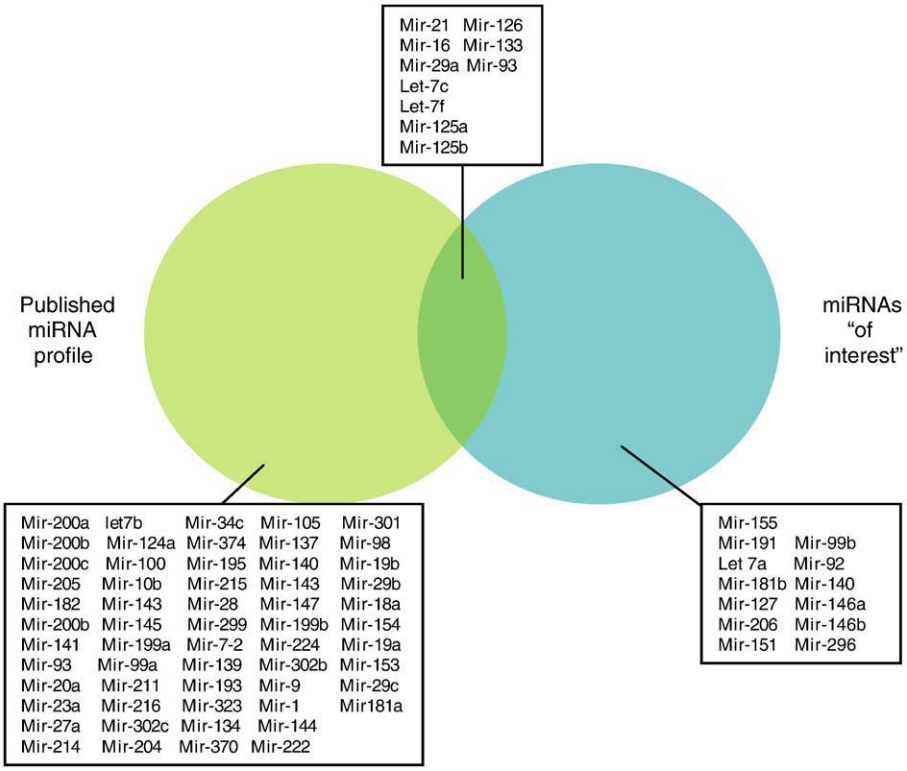

Fig. 1. Comparison of published miRNA profile and differentially expressed miRNAs from ovarian cancer patient serum.

126, $p=.0002$, $p=.003$, $p=.0001$, $p=.0003$, $p=.007$) and 3 miRNAs were under-expressed (mir-127, 155 and 99b, $p=.0001$, $p=.0003$, $p=.0001$) in the serum of ovarian cancer patients compared to controls. Fold-differences in median expression of the miRNAs in patients versus controls are demonstrated in Fig. 2.

Three patients were identified with pre-operative CA-125<35 U/ml. We then examined the three miRNAs with the highest serum expression, miRs-21, 92 and 93 for expression patterns in those patients with normal CA-125 in order to determine if miRNA patterns mimicked CA-125 patterns. These three miRNAs were found to be significantly over-expressed in these patients when median expression in the patient population was compared to controls (Table 1).

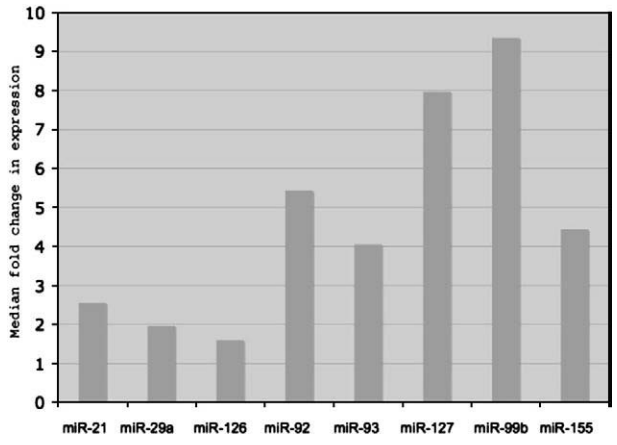

Fig. 2. Median fold-change differences in differentially expressed miRNAs between patient and control serum.

There was no correlation between miRNA status and grade, stage or histologic subtype. Due to the small sample size and recent diagnosis of disease we did not attempt to correlate miRNA status with progression-free interval or survival.

Discussion

We demonstrate that the extraction of RNA and identification of miRNAs from the serum of individuals diagnosed with ovarian cancer is feasible. We offer the first description of using a real-time PCR, microarray platform to screen large numbers of miRNAs while minimizing the amount of RNA needed. Additionally, we provide evidence that miRNAs may potentially serve as early detection biomarkers in patients with normal CA-125.

With our approach we were able to create a profile that was subsequently examined on a set of 19 patients and 11 healthy controls. Out of the 21 miRNAs of interest that we selected, 10 miRNAs were common to published ovarian cancer profiles. Among the 5 over-expressed miRNAs that we discovered are three potential oncomirs; mirs-21, 92 and 93. Over-expression of mir-21 has been demonstrated in glioblastoma, breast, colon, prostate, lung, pancreas and stomach cancers [19,20]. It has been shown to modulate expression of PTEN in hepatocellular carcinoma [10] as well as PDCD4 and maspin, two genes involved in regulating invasion and metastasis [21,22].

The most consistently over-expressed miRNA in serum from patients was miR-92. Mir-92a-1 is part of the mir-17–92

Table 1
miRNA over-expression in patients with normal pre-operative CA-125

ID	FIGO Stage	CA-125 (u/ml)	miR-21 Patient[a]/Control[b]	miR-92 Patient/Control[c]	miR-93 Patient/Control[d]
1050133	IIC	34	1.89/.79	27.5/4.3	2.37/.76
1050130	IV	13.4	1.54/.79	13.8/4.3	14/.76
1010026	IA	16.9	1.46/.79	16.1/4.3	3.5/.76

[a] Individual patient serum miRNA expression is defined as the 2(ΔCt).
[b] Inter-quartile range for median expression of miR-21 in controls: (.68–.93).
[c] Inter-quartile range for median expression of miR-92 in controls: (2.9–8.7).
[d] Inter-quartile range for median expression of miR-93 in controls: (.36–1.3).

polycistron, located on chromosome 13q13. A known oncomir, mir-17–92-enforced expression in a transgenic mouse model of lymphoma unequivocally demonstrated accelerated lymphoma progression [23]. Over-expression of miR-93 was associated with decreased progression-free and overall survival in ovarian cancer patients [13]. In gastric tumors, this cluster negatively regulated TGFβ tumor suppressor activities [24]. The proposed oncogenic activities of both mir-92 and mir-93 agree with our serum findings.

Contrary to the published ovarian cancer profiles, we have demonstrated significant over-expression of mir-29a and mir-126 in the sera from ovarian cancer patients. There have been a number of tumor suppressor activities proposed for both mir-126 and 29a. Mir-126 has been implicated as a "metastatic-suppressor" in breast cancer with loss associated with poor outcome [25]. Mir-29a has been found to be under-expressed in lung cancers; having been implicated in the modulation of methylation patterns seen in lung cancer [26]. While over expression of these miRNAs would tend to suggest they behave as oncomirs, TargetScan (4.2) does predict PTEN as a potential target of mir-29a. Whether or not these miRNAs exhibit predominant "tumor suppressor" or "oncogenic" activity remains to be seen.

Mir-127 has been identified as one of thirty-one down-regulated miRNAs in ovarian cancer cell lines [14]. It has recently been shown to be embedded in a CpG island and silenced completely in most cancer cell lines. In this same study, it was demonstrated that treatment of cell lines with 5-aza-2′-deoxycytidine not only restored mir-127 expression but also reduced expression of the proto-oncogene BCL6 [27]. Taken together these results identify mir-127 as a putative tumor suppressor gene, supporting our findings of decreased expression in patient serum.

Our study is hindered by the limited amount of published data regarding the extraction of quality miRNA from serum. While we did experience RNA degradation as well as genomic DNA contamination (results not shown), only 400 ng of total RNA are required for the TaqMan Array Human MicroRNA. Additionally, given that the amplicons of interest are approximately 25–30 nucleotides, we feel that some degradation of the RNA is tolerable. More importantly, the controls used in real-time PCR should account for both cross contamination by reagents (no-template control) as well as genomic DNA contamination (RT minus control). As microarray chips typically utilize up to 5 μg of sample, we feel that our real-time based approach will be superior until methods for extracting large amounts of pure RNA from serum are established. To our knowledge, this is the first description of utilizing this technology to obtain a miRNA profile on serum RNA.

Our sample size is small and lacks long-term outcome data. Additionally, it remains unclear whether these serum miRNAs are necessarily tumor-derived, associated with cellular contaminant or miRNAs involved in the host immune and/or stress response. While a number of miRNAs in our test set are important modulators of the immune response [28] the recent report by Taylor et al. [18] certainly provides proof of principle that serum-derived miRNA is a function of the miRNA signature at the tumor level. We present a pilot study demonstrating the potential utility of miRNAs as either biomarkers for further prospective evaluation in ovarian cancer detection studies or therapeutic targets.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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