Tumor and Stem Cell Biology

Microfluidic-Based Multiplex qRT-PCR Identifies Diagnostic and Prognostic microRNA Signatures in the Sera of Prostate Cancer Patients

Felix Moltzahn1-3, Adam B. Olshen4,6, Lauren Baehner1-3, Andrew Peek8, Lawrence Fong6,7, Hubert Stöppler8, Jeffry Simko5,6, Joan F. Hilton4,6, Peter Carroll3,6, and Robert Blelloch1-3,5,6

Abstract

Recent prostate-specific antigen–based screening trials indicate an urgent need for novel and noninvasive biomarker identification strategies to improve the prediction of prostate cancer behavior. Noncoding microRNAs (miRNA) in the serum and plasma have been shown to have potential as noninvasive markers for physiologic and pathologic conditions. To identify serum miRNAs that diagnose and correlate with the prognosis of prostate cancer, we developed a multiplex quantitative reverse transcription PCR method involving the purification of multiplex PCR products followed by uniplex analysis on a microfluidics chip to evaluate 384 human miRNAs. Using Δger8 and Δicer knockout (small RNA-deficient) mouse ES cells as the benchmark, we confirmed the validity of our technique and uncovered a considerable lack of accuracy in previously published methods. Profiling 48 sera from healthy men and untreated prostate cancer patients with differing CAPRA scores, we identified miRNA signatures that allow us to diagnose cancer patients and correlate with a prognosis. These serum signatures include oncogenic and tumor-suppressive miRNAs, suggesting functional roles in prostate cancer progression. Cancer Res; 71(2); 550–60. ©2010 AACR.

Introduction

One of 6 men will be diagnosed with prostate cancer during their lifetime (1). However, using current risk stratification strategies, it is still difficult to separate patients with rapidly progressing tumors, which require aggressive treatment, from those with indolent tumors. The result is overtreatment, leading to undesirable side effects for a large population of men and unnecessary consequential health care costs (2). Therefore, it is essential that new biomarkers are discovered, which can predict the outcome of the disease more accurately and, hence, enable better individual treatment decisions.

Cancer biomarkers provide a potentially powerful tool in the diagnosis/prognosis of cancer patients. In prostate cancer, the commonly used biomarker is prostate-specific antigen (PSA), which is measured in the blood and shows elevated levels in prostate cancer. Unfortunately, PSA alone is a poor predictor of disease outcome (3). Recently, microRNAs (miRNA) have been found in serum and have been proposed as potential biomarkers of normal physiology and disease (4). MiRNAs are short (18–24 nucleotides), noncoding RNAs that regulate gene expression posttranscriptionally by both destabilizing messenger RNAs (mRNA) and inhibiting their translation. Canonical miRNAs derive from longer polymerase II transcripts called pri-miRNAs. A complex consisting of the proteins DGC8R and Drosha process the pri-miRNAs to pre-miRNAs, which are then exported to the cytoplasm and cleaved by the protein Dicer to mature miRNAs. Exceptions to this processing include noncanonical miRNAs that bypass DGC8R/Drosha but are still being processed by Dicer. Knockout models of Δger8 and Δicer have been developed that remove only canonical miRNAs or both canonical and noncanonical miRNAs, respectively (5–7).

For prostate cancer tissue, there have been very few studies that compared miRNA expression levels in more than 10 benign and malignant samples (8–11). Noticeably, there is no overlapping subset between the down- or upregulated miRNAs (12, 13). Mitchell et al. (14) were the first to show a correlation between miRNAs found in plasma and the presence of prostate cancer. They showed that miRNAs originating from human prostate cancer xenografts can enter the circulation, be measured in plasma, and can robustly distinguish xenografted mice from controls. Furthermore, they found that serum levels of miR-141 distinguished patients

Authors' Affiliations: ¹The Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, ²Center for Reproductive Sciences, Departments of ¹Urology, ²Epidemiology and Biostatistics, and ³Pathology, ⁴UCSF – Helen Diller Family Comprehensive Cancer Center, and ⁵Division of Hematology and Oncology, Department of Medicine, University of California San Francisco, San Francisco, California; and ⁶Roche Molecular Diagnostics, Pleasanton, California

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Robert Blelloch, 513 Parnassus Avenue, HSE-1621, Box-D029, San Francisco, CA 94143. Phone: 415-476-2838; Fax: 415-476-1635; E-mail: blellochr@stemcell.ucsf.edu

doi: 10.1158/0008-5472.CAN-10-1229

©2010 American Association for Cancer Research.
with advanced prostate cancer from healthy controls (14). Lodes et al. (15) screened 21 sera from 5 different cancer entities (colon, ovarian, breast, lung, and prostate). In total, 6 prostate cancer patient samples were studied, of which 5 were from patients in advanced prostate cancer stages (3 and 4), and only 1 sample was from a nonadvanced stage. Four of the total 6 prostate cancer patients in this study had received systemic chemotherapy (Taxotere or Lupron with/without the bisphosphonate Zometa). Therefore, it remains unclear whether the altered serum miRNA levels are a result of the chemotherapy or reflect the actual advanced cancerous disease (15).

To establish a method for identifying miRNA signatures that could provide diagnostic and prognostic information for prostate cancer patients prior to any treatment, we developed a novel multiplex quantitative reverse transcription PCR (qRT-PCR) method and tested 4 sets of patients (12 patients in each set) with differing risk-stratifying “cancer of the prostate risk assessment” (CAPRA) scores. The method was validated using wild-type mouse ES cells (mESC) and Dgcr8 knockout and Dicer knockout mESC as negative controls, showing considerably improved accuracy relative to currently published protocols. Using this technique on a microfluidic platform, we screened patient serum samples for the expression level of 384 miRNAs and uncovered miRNA signatures that diagnosed prostate cancer samples and correlated but did not exactly match patient CAPRA scores, suggesting that these signatures may have the potential to separate patients within the risk groups and provide additional prognostic information. Many of the miRNAs found to correlate with groupings have been shown to have oncogenic or tumor-suppressive functions in different cancer contexts and, therefore, altered miRNA levels in the serum may reflect functional roles within the tumors.

Materials and Methods

ES cell culture

Wild-type, Dgcr8−/−, and Dicer−/− mESCs were made and authenticated in the laboratory by Southern blots, mRNA, and miRNA expression analysis, as described previously (5, 16).

Patient samples

Human serum samples were obtained with informed consent, and studies were done under the aegis of the committee on human research (CHR)—approved protocols. Normal serum (500 μL) was obtained from 12 healthy male blood donors. Serum samples (500 μL) of UCSF prostate cancer patients representing different CAPRA scores were collected from 36 patients before surgery. The CAPRA index is a scoring scheme based on the patients’ age, PSA level, clinical tumor stage, Gleason score, and percentage of biopsy cores positive for cancer at diagnosis, which currently provides the best prognostic insight (17). Patients were distributed as follows: 12 low-risk (CAPRA score 1), 12 intermediate-risk (CAPRA score 4), and 12 high-risk [CAPRA score > 5, N+ (regional lymph nodes)] patients. At the time-point of blood collection, none of the patients had received any therapeutic medication or surgical intervention. Samples were stored at −80°C until use.

RNA extraction

mESC. Total RNA from wild-type, Dgcr8−/−, and Dicer−/− mESC was extracted using TRIzol (Invitrogen), and RNA concentration was quantified by the absorbance at 260 nm.

Human serum samples. RNA extraction followed a previously described modified mirVana PARIS kit protocol (14). After extraction, the solution was concentrated (5×) using a centrifugal filter device (Microcon; Milipore).

mirR-specific primer

The design of primers and probes was based on the previously published protocol of Tang et al., and details are provided in supplementary data (Supplementary Fig. 1; ref. 18). Libraries for the miRNA-specific reverse stem-loop (RSL-P) and forward primers (FP) as well as for the miRNA-specific probes were designed based on the miRNA sequences from the miRBase database (Version 11; refs. 19, 20) and purchased from IDT. The sequences for 677 different primers and probes can be found in supplementary data (Supplementary Table 1).

Reverse transcription

mESC. For each sample, a multiplex RT reaction containing 96 RSL-P was done. One hundred nanograms of total RNA was used for a 5-μL reaction. Final RSL-P concentration was 1 nmol/L. Reaction conditions: 16°C for 30 minutes followed by 60 cycles at 20°C for 30 seconds, 42°C for 30 seconds, 50°C for 1 second, and finally 85°C for 5 minutes to inactivate MMLV-RT.

Human serum samples. For each sample, 4 independent multiplex RT reactions, each containing 96 unique RSL-P, were done. Final RSL-P concentration was 2 nmol/L. Reaction conditions were the same as for mESC.

Pre-PCR

mESC. For each sample, a multiplex pre-PCR containing 96 FP was done. All 5 μL of RT-Product was used as a template for 27.5 μL of pre-PCR. Final FP concentration was 50 nmol/L. Reaction conditions: 95°C for 10 minutes and 55°C for 2 minutes, followed by 12 cycles of 95°C for 1 second, and 65°C for 1 minute.

Human serum samples. For each sample, 4 independent multiplex pre-PCR reactions, each containing 96 unique FP, were done. Final concentrations and reaction conditions were the same as for mESC.

Purification of pre-PCR product

Gel purification. Each pre-PCR product was run through a 10% native polyacrylamide gel. PCR product band was cut out and extracted in 0.3 mol/L NaCl cDNA was precipitated in ethanol, redissolved in nuclease-free water and stored at −80°C until use.

ExoSAP-IT purification. Five microliters of the pre-PCR product was mixed with 2 μL of ExoSAP-IT and incubated at 37°C for 15 minutes. For inactivation, the mix was in a second step, incubated at 80°C for 15 minutes.
Column purification. Quiagen MinElute spin columns were used to purify fragments ranging from 70 base pairs (bp) to 4 kb after pre-PCR, following the manufacturer’s protocol.

ExoSAP-IT + column purification. Column purification was done as described directly after the first step of ExoSAP-IT purification, skipping the inactivation step.

384-well TaqMan qRT-PCR. One microliter of each purified Pre-PCR product was used in a 10-μL single-plex reaction. All reactions were duplicated. Briefly 2× TaqMan Universal Master Mix (ABI), 1 μmol/L FP, 1 μmol/L Universal Reverse Primer, and 0.2 μmol/L TaqMan-Probe was used for each real-time PCR. All reactions were run and analyzed on an Applied Biosystems 7900 Real-Time PCR. Reaction conditions: 55°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 55°C for 1 minute.

miRNA expression screening using Fluidigm microfluidic high-throughput platform

Each chip profiled 48 samples in duplicate for the expression level of 96 different miRNAs. Loading buffer was mixed with 2.25 μL of each purified Pre-PCR product and 2× TaqMan Universal Master Mix (ABI). For the assays, 1 μmol/L FP, 1 μmol/L Universal Reverse Primer, and 0.2 μmol/L TaqMan-Probe were combined with loading reagent. Volume per inlet was 5 μL. Reaction conditions: 55°C for 2 minutes and 95°C for 10 minutes, followed by 40 reagents of 95°C for 15 seconds and 55°C for 1 minute. The real-time qPCR analysis software provides amplification curves, color-coded heat maps, and cycle threshold (Ct). Threshold and linear baseline correction were automatically calculated for the entire chip. Since experimental errors, such as inaccurate pipetting or contamination, can result in amplification curves that look very different from a typical amplification curve, all amplification plots were checked both computationally and manually.

Statistical analysis

The initial data for statistical analysis were the ΔCt values for each sample. Seventy of 384 miRNAs were positive in at least 1 set of patients and, therefore, went into the final analysis. There were 38 samples analyzed since some samples had to be excluded because of technical problems and red blood cell lysis. Of these, 9 were healthy controls, 9 had a low CAPRA score, 11 had an intermediate score, and 9 had a high score. The remaining data were normalized for every sample and multiplex by subtracting the corresponding median. Complete linkage hierarchical clustering with correlation as the distance metric was done using Gene Cluster 3.0. To compare expression levels between groups, the t test was used. To examine trends in expression across more than 2 groups, linear regression was used. Then, 12 top candidates from these analyses were validated using single-plex qRT-PCR. To adjust for multiple comparisons, P values were converted to q values, using the method of Benjamini and Hochberg (21). The cutoff for statistical significance was a q value of less than 0.05. Receiver operating characteristic (ROC) curves were generated using the binormal method and the web-based calculator for ROC curves. Area under the curves (AUC) were automatically calculated and provided with individual ROCs (22).

Results

Optimization of multiplex qRT-PCR

To identify miRNA signatures that diagnose and correlate with prostate cancer prognosis, we set out to establish a robust profiling method that could be used on patient sera. Since the levels of small RNAs in serum are likely to be very low, a multiplex RT-PCR–based quantification method was evaluated. RSL-P, FP, and TaqMan probes were generated for all annotated human miRNAs (ref. 20; miRBase Version 11). The method involves RT and pre-PCR reactions carried out in a multiplex reaction, followed by pre-PCR reactions, and finally, a uniplex qPCR (ref. 23; Fig. 1). To test the accuracy of the published method, we performed multiplex qRT-PCR for 57 miRNAs of a 96-plex reaction that we, and others, have shown by microarrays and/or deep sequencing to be expressed in mESC (16, 24). Levels in wild-type ESC were compared with levels in Dicer knockout (lacking canonical miRNAs) and Dicer knockout (lacking canonical and noncanonical miRNAs) cells, serving as optimal negative controls. Surprisingly, although many miRNAs showed lower levels in knockout cells as expected, several expressed miRNAs were not detected at all and some even showed increased levels in the knockouts relative to wild-type ESC (Fig. 2A). This finding suggested a lack of accuracy in the previously published multiplex qRT-PCR technique.

We hypothesized that this lack of accuracy could be due to the carry-over of the many primer sets from the multiplex pre-PCR step to the uniplex qPCR reactions. To test this hypothesis, pre-PCR products were purified away from primers, following pre-PCR, by size selection on native polyacrylamide gels (Fig. 1 and Supplementary Fig. 2). As expected, bands for the expected product sizes were seen in the wild-type but not in the Dicer knockout ESC (Fig. 2A). This finding suggested a lack of accuracy in the previously published multiplex qRT-PCR technique.

The purified product was then subjected to uniplex qPCR reactions. The resulting quantitation revealed a remarkable improvement in accuracy of the modified protocol over the standard protocol. Of 53 canonical miRNAs, 16 miRNAs were additionally detected, showing substantially decreased levels in Dicer knockout ESC, relative to wild-type cells. Surprisingly, some miRNAs were not detected independent of the technique used. No canonical miRNAs showed an increase in the Dicer knockout cells, unlike the standard protocol. All 3 Dicer- and Dicer-independent, noncanonical miRNAs (ref. 16; miR-320, -484, and -877) showed little change or even decreases in the Dicer knockout (lacking canonical miRNAs) cells, serving as optimal negative controls.
Step 1: multiplex reverse transcription of 96 target microRNAs using 96 different and specific reverse stemloop primers.

Step 2: multiplex pre-PCR using 96 different and specific forward primers and universal reverse primers.

Step 3: singleplex qRT-PCR

A. Without purification
Preamplification product including carry-over of primer sets and primer-dimers.

or

B. With purification
Preamplification product after purifying away excessive primer-sets.

Figure 1. Diagrammatic step-by-step description of multiplex qRT-PCR miRNA expression profiling with and without purification after pre-PCR. Excessive primer from multiplex reverse transcription (step 1) and multiplex preamplification (step 2) are removed before real-time detection (step 3), resulting in a purified cDNA template for qRT-PCR. FP, miRNA-specific forward primer; URP, universal reverse primer; R-SLP, miRNA-specific reverse stem-loop primer.
qPCR vastly improves the robustness of the multiplex qRT-PCR protocol.

Alternative purification methods

To make the purification approach applicable for large sample sets, we tested alternative methods of removing the carry-over of primers. ExoSAP-IT successfully removed primers but also led to a partial degradation of the PCR product (Supplementary Fig. 3A, lane 2). We hypothesized that degradation occurred during the heat inactivation of the exonuclease since the short PCR products would likely begin to partially denature as the temperature increased. Therefore, we avoided heat inactivation and directly purified the PCR products from the enzymatic reaction using spin columns (Qiagen MinElute). Under these conditions, primers were removed without any discernible PCR product degradation (Supplementary Fig. 3A, lane 3). Importantly, qRT-PCR results using the purified product showed similar Ct values and similar

![Figure 2. Purification after preamplification greatly improves the accuracy of the standard multiplex qRT-PCR protocol. A, qRT-PCR data with and without gel-based purification. Shown are expression levels of small RNA-deficient (Dgcr8/−/−) relative to wild-type mESCs for 57 miRNAs out of a 96-plex reaction with (black) and without (light gray) an additional purification step after preamplification. Purification results in the detection of more miRNAs as well as in the loss of false-positive signals in the Dgcr8/−/− background. B, qRT-PCR data for noncanonical (Dgcr8-independent) miRNAs with (black) and without (light gray) gel purification. Similar or even increased expression levels in the Dgcr8 knockout background relative to wild-type cells show the correct identification of Dgcr8-independent miRNAs. Increased levels are likely secondary to the stabilization of noncanonical miRNAs in the absence of canonical miRNAs (16). C, qRT-PCR data comparing expression levels in Dgcr8/−/− (black) and Dicer−/− (light gray) mESC relative to wild-type after gel purification. Results show a similar decrease for canonical miRNAs in both Dgcr8 and Dicer mESC, the absence of false-positive signals, and the proper categorization of rare Dgcr8-independent/Dicer-dependent small RNAs.](#)
relative expression values of small RNA-deficient cells, as well as the ability to correctly classify noncanonical miRNA as seen after gel purification (Supplementary Fig. 3A and C), suggesting equal robustness of this simplified purification approach.

Global profiling of untreated prostate cancer patients with differing prognostic risk

Having established a robust multiplex qRT-PCR method, we initiated profiling experiments on patient sera representing different risks of disease progression. All sera samples (healthy, low-, intermediate-, and high-risk patients) were collected prior to any disease-related medication or clinical intervention. Patient data are summarized in Table 1.

Isolated serum RNA underwent RT and pre-PCR with conventional PCR machines. After gel purification, quantitative single-plex PCRs were done on the Fluidigm Biomark platform $96 \times 4$ arrays. Each of the total 4 arrays was loaded with 96 miRNA TaqMan assays ($96 \times 4 = 384$ assays) and 96 RNA samples (48 patients $\times 2 = 96$ samples) totaling 9,216 reactions per array with each reaction being 6 nL. The $\Delta$Ct values were median-normalized before analysis. For each group, 9 samples (11 in the intermediate-risk group) went

### Table 1. Pre- and postinterventional patient data

<table>
<thead>
<tr>
<th></th>
<th>Prostate carcinoma patients</th>
<th>Healthy blood donor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAPRA 1</td>
<td>CAPRA 4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>48–71</td>
<td>51–72</td>
</tr>
<tr>
<td>Mean</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td><strong>PSA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.4–5.7</td>
<td>2.4–16.1</td>
</tr>
<tr>
<td>Mean</td>
<td>4.33</td>
<td>6.88</td>
</tr>
<tr>
<td><strong>Stage, clinical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1c</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>T2a</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>T2b</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>T2c</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><strong>Gleason sum biopsy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6</td>
<td>6–8</td>
</tr>
<tr>
<td>Mean</td>
<td>6</td>
<td>7.3</td>
</tr>
<tr>
<td><strong>Stage, pathology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2a</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>pT2b</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>pT2c</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>pT3a</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>pT3b</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Gleason sum postsurgery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>5–7</td>
<td>6–7</td>
</tr>
<tr>
<td>Mean</td>
<td>6.33</td>
<td>6.91</td>
</tr>
<tr>
<td>LN+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Follow-up</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td><strong>Follow-up days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>203–2,183</td>
<td>222–1,599</td>
</tr>
<tr>
<td>Mean</td>
<td>793</td>
<td>872</td>
</tr>
<tr>
<td><strong>Progression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Yes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>PCA medication</strong></td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; M, metastases.
into analysis since some samples had to be excluded because of technical problems and red blood cell lysis. Seventy of 384 miRNAs were present in all sera, and therefore, comparable between samples. Normalized absolute expression levels of all the expressed miRNAs were used for complete hierarchical clustering with Cluster 3.0. The unsupervised analysis segregated 8 of 9 healthy patients into one cluster of size 9. There was also a trend toward clustering by CAPRA group (Fig. 3A). These findings suggest that miRNA signatures can both diagnose prostate cancer as well as segregate the risk for progression.

**Trends for individual miRNAs**

To identify individual miRNAs from the screen that showed the most significant changes between healthy versus all malignant and healthy versus high-risk cancer sera, a pairwise comparison using the \( t \) test was done. This analysis identified 12 statistically significant candidates (miR-19a, -20b, -24, -26b, -30c, -93, -106a, -223, -451, -874, -1207-5p, and -1274a; expression levels relative to healthy samples shown in Fig. 3B). Ten miRNAs (miR-20b, -24, -26b, -30c, -93, -106a, -223, -451, -874, -1207-5p, and -1274a) were substantially different between the healthy and all malignant samples. Four were downregulated in the cancer group (miR-223, -26b, -30c, and -24), and 6 were upregulated in the cancer group (miR-20b, -874, -1274a, -1207-5p, -93, and -106a). MiR-19a and -451 were significantly different between the healthy versus high-risk group. To examine trends across all groups and in cancer patients alone, linear regression was used and identified an important linear trend for miR-106a and miR-93 across all groups as well as among the cancer patients alone. For miR-24, an important linear trend among cancer patients was observed but not across all groups.

All 12 top candidates from the screening were then validated by individual qRT-PCRs and statistically analyzed the same as the screening results. For miR-19a, there was weak correlation between the screening and follow-up PCR results. For miR-20b, the expression levels were higher in the cancer group versus the healthy group in the follow-up PCR, but the result was not statistically significant (Supplementary Fig. 4 and Table 2). In 10 cases (miR-24, -26b, -30c, -93, -106a, -223, -451, -874, -1207-5p, and -1274a), Fluidigm and individual PCR data produced statistically significant changes in the same direction (Supplementary Fig. 4). Specifically, miR-223, -26b, -30c, and -24 were downregulated in the cancer group, and miR-874, -1274a, -1207-5p, -93, and -106a were upregulated.

Among the 10 miRNAs, there were 7 distinct patterns (Figs. 3B and 4). Three miRNAs (miR-223, -874, and -1207-5p) showed substantially altered expression after the transition from healthy to cancer but stable expression within the different risk-stratified cancer sera. In particular, miR-223 showed a stable decrease, whereas miR-1207-5p and -874...
showed a stable increase in the cancer patients relative to healthy controls (Fig. 4A and B). Two miRNAs showed a linear relationship between miRNA levels and cancer risk: miR-24 steadily decreased with risk, whereas miR-106a steadily increased with risk (Fig. 4C and D). Two miRNAs (miR-26b and -30c) were down in the low- and intermediate-risk groups relative to both healthy controls and metastatic disease (Fig. 4E). A stepwise increase from healthy to low- and intermediate-risk and again to metastatic disease was observed for miR-93 and -1274a (Fig. 4F). The final trend was an increase for miR-451 in the patients with metastatic disease with little to no change in low- and intermediate-risk groups (Fig. 4G). P values were determined, and the data were adjusted for multiple comparisons (q values; Table 2).

The 10 miRNAs were also evaluated for potential correlations with individual variables of the CAPRA score among the cancer patient samples, including PSA, Gleason, and age (Supplementary Fig. 5A–C). Although some of the miRNAs showed trends with these variables, none reached a level of significance.

### Diagnostic utility of miRNA serum profiling
To further explore the diagnostic ability of the signature, ROC curves were created for individual miRNAs. Testing the ability to discriminate healthy from cancer serum, the binormal method was used to create ROC curves, and AUCs were calculated as a measurement of the accuracy. The curves and AUCs (0.778, 0.812, 0.845, 0.876, 0.907, 0.928) showed a good performance of the individual miRNAs to classify healthy and malignant samples (Supplementary Fig. 6).

### Prognostic utility of miRNA serum profiling
The uncovered miRNAs showed distinct differential expression levels for the different risk-stratified groups (Fig. 4). Furthermore, the analysis of the unsupervised clustering of the individual serum samples also trended to, but not unambiguously differentiated, the individual risk of progression groups (Fig. 3, Supplementary Fig. 7). The strong trends of serum miRNAs to correlate with the different risk groups indicate a promising prognostic potential; however, there remained pronounced variability within the different groups (Fig. 5). Any marker that could provide additional prognostic information beyond that of the CAPRA score should show variability within the CAPRA-defined groups. It would be most important to be able to identify patients in the low-risk group that are actually at high risk for progression as well as those in the intermediate-risk group that are actually unlikely to progress. Three miRNAs, miR-93, -106a, and miR-24, showed consistently low and high levels among the healthy and metastatic groups while showing variability within the low- and intermediate-risk groups. This in-group variation in the low- and intermediate-risk groups may provide additional information to current scoring indexes. However, large-scale longitudinal studies will be required to make a conclusive determination. Such studies would additionally be likely to identify new informative miRNAs.

### Discussion
Here, we describe an optimized multiplex qRT-PCR approach, which is used in combination with a microfluidics platform and enabled us to screen 48 prostate cancer patient sera for alterations in miRNA levels. Although previous smaller-scale studies suggested the utility of plasma miRNAs in prostate cancer (14, 15), this is the first study to evaluate miRNA levels in an untreated patient set with different risks of progression based on the leading risk indicator currently available. Identifying prostate cancer patients at risk for progression prior to treatment is an essential goal since...
current measures lead to an overtreatment of some patients and undertreatment of others (2, 3).

miRNA signatures uncovered in this study not only distinguished sera between healthy and high-risk but also between healthy and curable disease stages (low- and medium-risk) sera. Cluster and ROC analyses underscored the diagnostic potential of our serum miRNA signatures. Importantly, the expression levels of the miRNA signatures also trended with CAPRA scores and were able to cluster samples in accordance with their CAPRA score, demonstrating the ability to further

Figure 4. Patterns of significantly altered individual miRNAs. Candidates were validated by individual qRT-PCR, and 7 distinct patterns were observed for the significantly altered individual miRNAs. Results are shown relative to expression levels in healthy samples, including the standard error. A, downregulated in all cancer groups. B, upregulated in all cancer groups (note the discontinuous scale). C, incremental decrease with increasing risk. D, incremental increase with increasing risk. E, down in low- and intermediate-risk groups relative to healthy and metastatic. F, increase in low- and intermediate-risk groups and further increase in the high-risk group. G, predominant in metastatic disease. Note that miR-20b is not statistically significant in the follow-up PCR (Table 2). Relat., relative; interm., intermediate.
separate cancer patients according to their risk of recurrent cancer. Interestingly, the in-group variation indicated that serum miRNAs may have the potential to separate patients within the risk categories and may, therefore, provide additional information. Investigating the potential of serum miRNAs to predict the clinical outcome and cancer behavior of prostate cancer patients in large-scale clinical trials will be required to confirm such a proposition.

Our modified qRT-PCR demonstrates that primer carry-over from the multiplex PCR has detrimental effects on miRNA quantification. Purification of the PCR product and, therefore, removing multiplex primers prior to quantitative PCR led to (i) the detection of more miRNAs (increased sensitivity), (ii) the loss of false-positive signals in Dgcr8−/− and Dicer−/− backgrounds (increased accuracy), and (iii) the proper categorization of rare Dgcr8-independent/Dicer-dependent small RNAs. Using this technique on a nanoliter scale on a microfluidic chip allows for high-throughput multiplex qRT-PCR in a timely and cost-effective manner. In addition, we provide sequences for 677 different miRNA-specific primers and probes as well as a detailed description of how primers were designed. Therefore, this approach can be easily adapted by others, allowing for the screening of any samples of interest, even when only small quantities of input RNA are available.

The miRNAs found in our study may have functional roles in prostate cancer. Indeed, all have been suggested to have either oncogenic or tumor-suppressive roles in different settings. For example, miR-24 mediates the inhibition of the cell cycle via the suppression of cell cycle–control genes like E2F2 and MYC (25). miR-106a, -20b, and -93 are part of the miR-17 family, which have been shown to promote tumorigenesis in various models (26–28). Noticeably, in accordance with our findings, miR-106a and miR-30c seem to be overexpressed in prostate cancer tissue samples compared with healthy prostate tissue (8). Furthermore, miR-93 is upregulated in prostate

Figure 5. Inter- and intragroup variability. The miRNAs trend to correlate with the different risk groups; however, the remaining in-group variability could provide additional information to current scoring indexes. interm., intermediate.
cancer tissue along with its host gene *MCM7* (29, 30). Based on these associations, it will be important to determine the cell source and function of the miRNAs that we find elevated and depressed in the sera of prostate cancer patients of different risk for progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

Acknowledgments

We thank the members of the Blelloch lab for critical reading; June Chan for help with patient samples and clinical data; Nathan Hankapillar for advice on preparing RNA from serum; Mark Ansel for suggestions with alternative purification; and Alain Mir, Caroline Dando, and Griffen Lauer from Fluidigm for providing technical assistance and machine access.

Grant Support

NIH/National Institutes of Neurologic Disease (K08 NS48118 and R01 NS057221), California Institute of Regenerative Medicine (New Faculty Award RN2.00906), and the Pew Charitable Trust (R. Blelloch). Cancer Center Support grant P30 CA82103 NIH-NCI (A.B. Olshen) and the Wissenschaftlich Urologische Gesellschaft eV (F. Moltzahn).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 9, 2010; revised November 8, 2010; accepted November 15, 2010; published OnlineFirst November 22, 2010.

References

Microfluidic-Based Multiplex qRT-PCR Identifies Diagnostic and Prognostic microRNA Signatures in the Sera of Prostate Cancer Patients

Felix Moltzahn, Adam B. Olshen, Lauren Baehner, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1229

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/11/22/0008-5472.CAN-10-1229.DC1

Cited articles
This article cites 28 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/2/550.full.html#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/71/2/550.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.