Investigation of miR-21, miR-141, and miR-221 expression levels in prostate adenocarcinoma for associated risk of recurrence after radical prostatectomy

Qizhi Zheng², Sarah B. Pesko³, Judit Ribas¹†, Fatema Rafiqi¹, Tarana Kudrolli¹, Alan K. Meeker¹,²,⁴, Angelo M. De Marzo¹,²,⁴, Elizabeth A. Platz¹,³,⁴, and Shawn E. Lupold¹,⁴*

¹ The James Buchanan Brady Urological Institute and Department of Urology, Johns Hopkins School of Medicine, Baltimore, MD.
² Department of Pathology, Johns Hopkins School of Medicine, Baltimore, MD.
³ Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.
⁴ Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD.

†Current affiliation: Pharmacology Unit, Department of Experimental Medicine, School of Medicine, University of Lleida, Catalonia, Spain

*Correspondence: slupold@jhmi.edu; 600 N Wolfe St, Park 209, Baltimore, MD 21287 Phone: 410-502-4822, FAX: 410-502-7711

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DISCLOSURE STATEMENT
The authors have nothing to disclose.
ABSTRACT

BACKGROUND. MicroRNAs (miRNAs) are small non-coding RNAs that regulate a broad array of cellular and disease processes. Several miRNAs are differentially expressed in cancer and many are being considered as biomarkers for predicting clinical outcomes. Here we quantified the expression of three miRNAs, miR-21, miR-141, and miR-221, from prostate cancer surgical specimens and evaluated their association with disease recurrence after primary therapy.

METHODS. A pilot nested case-control study was designed from a large cohort of men who underwent radical prostatectomy between 1993 and 2001. Total RNA was extracted from malignant prostate tissue of 59 cases (recurrence) and 59 controls. Cases and controls were matched on age, race, pathologic stage and grade. The relative expression of each miRNA was then determined for each sample by quantitative real-time RT-PCR. Conditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) of recurrence for tertiles of miRNA expression. We noted block storage time effects and thus, used separate tertile cutpoints based on the controls by calendar year of prostatectomy.

RESULTS. Lower miR-221 expression was associated with a higher risk of recurrence; the ORs were 3.21 for the lowest tertile and 2.63 for the middle tertile compared with the highest tertile of expression (P-trend=0.02). This pattern was unchanged after multivariable adjustment (P-trend=0.05). No statistically significant trends were observed for miR-21 or miR-141 after multivariable adjustment.

CONCLUSIONS. Based on this small pilot study, men with localized prostate cancers with lower miR-221 expression may have a greater risk for recurrence after surgery.
INTRODUCTION

MicroRNAs (miRNAs) are a functional class of small non-coding RNAs that regulate gene expression [1]. Mature miRNAs are generated in the cytoplasm, where they bind to target mRNAs by hybridization with partial sequence complementarity. This interaction results in gene silencing through mRNA degradation and/or inhibition of mRNA translation. There are over 1,800 miRNAs in the human genome and many have been found to regulate a diversity of important cellular and disease pathways, including cancer. The experimental over-expression or inhibition of specific miRNAs in cellular and animal models can result in tumorigenesis, or in more aggressive cancer phenotypes, indicating the potential of miRNAs to function as oncogenes and tumor suppressors [2]. During cancer development and progression, miRNA expression patterns become highly altered and can be associated with the developmental lineage of the cancer and of the disease state [3]. Consequently, specific miRNAs or groups of miRNAs are being considered as prognostic biomarkers and/or therapeutic targets for the future management of cancer [4].

Differential gene expression patterns have the potential to accurately define tumor types and subtypes, to predict risk of recurrence or response to therapy and to guide the development of new therapies [5]. This is of particular importance to prostate cancer, where the majority of men diagnosed with the disease will not progress to aggressive disease or death within their lifetime [6]. At the same time over 29,000 men will die from metastatic castration resistant prostate cancer each year, making it the second leading cause of cancer death in American men [7]. Therefore, there are unmet needs regarding the calculated risk for newly diagnosed patients and for the treatment of advanced prostate cancer patients. There are several sources of tissue available for prostate cancer risk analyses throughout the treatment period, including biopsy and
radical prostatectomy specimens, as well as circulating tumor cells and bone marrow biopsies. Other fluids such as blood, urine and prostatic secretions are also excellent sources for potential risk markers. Each of these has been widely examined for the presence of altered proteins and gene transcripts.

A number of miRNAs have been found to be differentially expressed in malignant tissues from prostate cancer surgical specimens [8-12]. Similarly, differential miRNA gene expression patterns have been detected in the serum of men with advanced prostate cancer [13-16]. Collectively, these and other studies have identified a number of miRNAs that are commonly deregulated in prostate cancer. Here we have focused on three prostate cancer associated miRNAs: miR-21, miR-141 and miR-221. Several previous studies have found these miRNAs to be differentially expressed in prostate cancer; however their association with biochemical recurrence has been less clear [17-25]. To address these discrepancies, we conducted a pilot study to quantify the expression levels of these miRNAs in radical prostatectomy specimens from 118 men who underwent surgery at Johns Hopkins. These tissues were sampled from a larger nested case-control study designed to evaluate the association of potential risk markers for prostate cancer recurrence after therapy [26]. Malignant prostate tissue from cases (recurrence) and controls (non-recurrence) from this population were matched for age, race, pathologic stage, and grade to determine their associations beyond accepted risk and prognostic factors. The results of this nested case-control study provide new data regarding the association of miR-21, miR-141 and miR-221 with prostate cancer aggressiveness and their potential use as prognostic markers in primary tumor tissue. The reduced expression of one of the prostate cancer associated miRNAs, miR-221, was found to be associated with recurrence.
METHODS

Study Design and Population.

We conducted a pilot study of 59 men who experienced recurrence after prostatectomy and 59 matched men who did not (controls). We sampled malignant prostate tissue from these men from a nested case-control study that we previously designed specifically to evaluate risk factors for, and biomarkers of, recurrence. The full nested case-control study includes all 524 recurrence cases and a sample of 524 controls identified among 4,860 prostate cancer patients who underwent radical retropubic prostatectomy for clinically localized prostate cancer at Johns Hopkins in Baltimore, MD between 1993 and 2001. We did not include men who received hormonal or radiation therapy before their prostatectomy or men who received hormonal adjuvant therapy before their recurrence. These men were followed for outcome through 2004. Recurrence was defined as biochemical recurrence (serum PSA >0.2 ng/mL), local recurrence, systemic metastases or death from prostate cancer. For each case we used incidence density sampling to select a control who had not experienced recurrence by the date of the case’s recurrence, and who was similar to the case on age at surgery, race, pathologic stage, and Gleason sum in the prostatectomy specimen [27]. Tissue microarrays and germline DNA have been prepared for the full nested case-control study. For this pilot we selected malignant prostate tissue from men included on the first two tissue microarrays, with the goal of achieving a sample size of at least 50 matched pairs (final number 59 matched pairs). We have previously used this set to evaluate other markers of prognosis [26,28,29].

Tissues and RNA extractions. Tumor cores from formalin fixed paraffin embedded (FFPE) tissues were obtained for the 118 men from the Department of Pathology at the Johns Hopkins
School of Medicine. Two 0.6 mm cores were punched from an identified tumor area for total RNA isolation. RecoverAll Total Nucleic Acid Isolation kit from Ambion (Grand Island, NY) was used for RNA isolation. In brief, samples were deparaffinized twice with xylene at 50°C for 5 min each, and then twice by 100% ethanol. After air drying, samples were digested with protease at 50°C for 16 hours. The next day, samples were heated to 80°C for 15 minutes and the RNA isolation was completed according to the manufacture’s instruction. The quantity and quality of the RNA samples were determined by Nanodrop (Thermo Scientific, Wilmington, DE), then stored RNA at -80°C.

Quantitative RT-PCR. Reverse transcription (RT) of RNA was performed by using Taqman MicroRNA Reverse Transcription Kit from Applied Biosystems (Grand Island, NY). RNA from three cell lines know to express each miRNA (MCF-7 for miR-21; PC3 for miR-221; and LNCaP for miR-141) were five-fold serial diluted from 2ng/µl to 0.016ng/µl and utilized as references for assay linearity and efficiency. 2ng/µl RNA from FFPE tissue was used for RT. 5µl from each sample was utilized for each miRNA and U6 snRNA. The RT reaction was 16°C for 30 minutes, 42°C for 30 minutes, followed by heat inactivation at 85°C for 5 minutes. QRT-PCR probe and primers were obtained from Applied Biosystems. All PCR reactions were performed using Bio-Rad MyiQ single color Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The mean Ct values for U6 were 23.61-25.58 and the mean Ct values for miR-141, miR-21, and miR-221 were 28.19-28.21, 24.32-25.09, and 26.72-27.10, respectively. The relative standard curve method was applied using diluted cell line materials. Each standard curve contains 4 points in triplicate (5-fold serially diluted cell line RNA, see above) and were optimized for efficiencies of 100% ± 5%. All qPCR reactions and thermal
cycling conditions followed manufacturer’s instruction. Briefly, holding at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Relative normalized target gene quantity (SQ) was determined from standard curves with U6 snRNA as a reference gene for normalization.

**Statistical Analysis.**

Differences in characteristics between the cases and their matched controls were evaluated using nonparametric tests (Wilcoxon sign rank test or McNemar’s test). No Ct values were excluded from the analysis. Relative normalized expression levels of the three miRNAs were not normally distributed. Thus, we calculated medians and interquartile ranges, and tested for case-control differences in their expression using the Wilcoxon sign rank test. We calculated odds ratios of recurrence and 95% confidence intervals by tertile of miRNA expression level using conditional logistic regression, taking into account the matching factors (age, race, stage, and grade) and adjusting for preoperative serum prostate specific antigen concentration, calendar year of surgery, surgical margins status and residual difference between the cases and controls in the matching on pathological stage. Because we noted tissue block storage time effects (Supplementary Figure S1), we calculated tertile cutpoints based on the miRNA expression distributions among the controls separately by calendar year (early: 1993-1994, late: 1995-2000). We used the highest tertile of miRNA expression as the reference group. We tested for trend across the tertiles by entering a single ordinal variable with possible values of 1, 2, and 3 corresponding to the lowest to highest tertiles of miRNA expression. Quality control analyses were also performed to determine whether differences in U6 quantities or miRNA Ct values between cases and controls influenced the results. No significant bias was found. All analyses
were performed using SAS version 9.2 (SAS Institute, Cary, NC, USA). Statistical tests were two-sided, and $P$-values $< 0.05$ were considered to be statistically significant.

**RESULTS**

By design, cases and controls were similar on the matching factors of age at surgery, race, and pathologic stage and Gleason sum in the radical prostatectomy specimens (Table 1). Cases were more likely to have positive surgical margins than controls, but they did not differ on presurgery PSA concentration (Table 1). In the malignant tissue of both cases and controls, miR-21 expression level was highest, followed by miR-141 and then miR-221 (Table 2). Without taking into account tissue block storage time, expression levels of all three miRNAs were statistically significantly lower in the malignant tissue of cases than in controls (Table 2).

We observed that the relative normalized expression levels of all miRNAs were higher, in both cases and controls, for prostatectomy samples from earlier calendar years (Supplementary Figure S1). Therefore, we calculated tertile cutpoints based on the miRNA expression distributions among the controls separately by calendar year. Early cases are considered those from radical prostatectomies performed in the years 1993 to 1994, and late cases are considered those from surgeries performed in the years 1995-2000. Men in the lowest tertile of expression of miR-221 had a higher risk of recurrence when compared to men in the highest tertile ($P$-trend=0.02; Table 3). The pattern of association was unchanged for miR-221 after further adjustment ($P$-trend=0.05). No statistically significant trends were observed for miR-21 or miR-141 after multivariable adjustment (Table 3). The log expression of each miRNA in the malignant tissue of cases and controls, separated by year of surgery, is presented in Figure 1.
DISCUSSION

The primary goal of this small pilot study was to evaluate the association of miR-21, miR-141 and miR-221 expression levels in prostate cancers with disease recurrence following radical prostatectomy. The study was specifically designed using matched cases and controls to minimize the influence of known prognostic factors for prostate cancer recurrence, such as stage, grade and age. This approach can emphasize biology that is independent of Gleason histologic pattern, which has served as one of the most powerful predictors of disease progression for decades. Our study included a subset of the case-control pairs that have been previously utilized to study the association of Cyr61, PTEN, SPARCL1, ERG, and TMPRSS2-ERG fusion with prostate cancer recurrence through immunohistochemical (IHC) or fluorescence in situ hybridization (FISH) staining [28-31]. We originally sought to use a similar approach, microRNA in situ hybridization (ISH), to quantify miRNA expression in these Tissue Microarrays (TMAs). However, we were unable to reliably detect miRNAs by ISH with archived FFPE human tissues. We therefore designed a smaller pilot study from the original nested case-control samples and quantified the expression level of each miRNA by real-time RT-PCR analysis using malignant tissue cores isolated from the FFPE tissue blocks. The results suggest that the expression of one of the three miRNAs, miR-221, is associated with prostate cancer recurrence in a dose-dependent manner.

miR-221 has been the subject of multiple miRNA studies in clinical specimens. In several cancers miR-221 levels are reported to be elevated, when compared to healthy tissues [32-35]. However, in prostate cancer, miR-221 expression levels have been universally reported to be decreased when compared to non-malignant tissues [20,21,36,37]. On the other hand, there have been conflicting reports regarding the association of miR-221 expression levels with prostate
cancer aggressiveness. Several reports have found miR-221 levels to be decreased in prostate cancers which later recurred [18,19,21], whereas others have found no significant association of miR-221 with disease recurrence [17,20,22]. Here, in our pilot study, we observed that miR-221 levels were significantly decreased in the cancers of men who later recurred when compared to controls, and that the risk of recurrence increased with decreasing miR-221 expression. This new data provides additional support for miR-221 as a potential prognostic marker and therapeutic target for higher risk prostate cancer. Further evaluation is required in larger studies to confirm these conclusions.

miR-141 was first reported as a potential biomarker for prostate cancer due to its elevated levels in the serum of patients with prostate cancer when compared to healthy controls [13]. Several other studies have further found that miR-141 expression levels are elevated in prostate tumors [16,36,38]. In a recent screen to identify differentially expressed miRNAs as markers for prostate cancer biochemical failure after primary therapy, miR-141 was found to be a top candidate [24]. However, the expression of miR-141 alone was not sufficient to accurately predict biochemical failure as an independent marker. The new results from our study provide additional data regarding miR-141 expression and risk of disease progression. While the risk of recurrence appeared to be higher in men with the lowest miR-141 expression, this association was not statistically significant and no dose response was present across the tertiles of expression after multivariable adjustment. Taking our pilot study and the literature together, there is faint evidence that differential miR-141 expression may be associated with risk of recurrence, but further studies are required to validate or negate these observations.

The final miRNA evaluated in our analysis was miR-21, which is one of the most highly studied microRNAs in cancer. miR-21 was originally identified as the most commonly
upregulated miRNA in multiple cancer types, including prostate [8]. Other studies have also reported miR-21 levels to be increased in prostate cancer patient tumors, serum, and plasma when compared to controls [8,39-41]. However, the association of miR-21 levels and prostate cancer aggressiveness is less clear. Li and colleagues found miR-21 positivity, by *in situ* hybridization in cancer specimens, to be associated with recurrence after radical prostatectomy [23]. In a separate study, a higher proportion of recurrent prostate cancers was found to have lower, rather than higher, miR-21 expression when compared to non-recurrent cases [17]. In the same study Amankwah and colleagues reported reduced miR-21 to be an independent risk factor for recurrence in obese men [17]. Here, in our study, miR-21 expression level was not associated with recurrence after taking into account tissue block storage time. This was a surprising result because miR-21 has been shown to have oncogenic, growth and survival promoting properties in prostate and other cancer models [40,42-48]. We therefore anticipate that miR-21 may contribute to advanced disease, rather than local disease, which we had initially predicted [40]. Nonetheless, further studies are needed to correlate the expression and role of miR-21 in local, recurrent, and advanced disease.

**CONCLUSIONS**

In summary, we have studied the expression levels of three microRNAs in prostate cancer and their association with recurrence after radical prostatectomy. The matched study design was applied to emphasize the potential value of these biomarkers in the absence of other known prognostic markers for recurrence, such as stage and grade. These results add to the collective literature on these three microRNAs and their association with prostate cancer recurrence and disease aggressiveness. The results suggest that miR-221 expression level in
prostate cancer is inversely associated with recurrence. Further studies of this microRNA and its associated pathways may uncover new mechanisms for cancer progression and therapeutic intervention.

ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Distribution of miRNA expression by case-control status and calendar year of prostatectomy. Log transformed expression of (A) miR-221, (B) miR-21 and (C) miR-141 by case or control status and year of surgery (categorized into Early: 1993-1994 or Late: 1995-2000).

FIGURES AND TABLES
Table 1

Characteristics of prostate cancer recurrence cases and matched controls

<table>
<thead>
<tr>
<th></th>
<th>Recurrence Cases N=59</th>
<th>Controls N=59</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± standard deviation age (years)</td>
<td>58.5 ± 7</td>
<td>58.9 ± 6.3</td>
<td>Matched</td>
</tr>
<tr>
<td>White (%)</td>
<td>86.4</td>
<td>91.5</td>
<td>Matched</td>
</tr>
<tr>
<td>Positive surgical margins (%)</td>
<td>35.6</td>
<td>20.3</td>
<td>0.06</td>
</tr>
<tr>
<td>Median (interquartile range) pre-surgery PSA concentration (ng/mL)</td>
<td>8.0 (6.3, 12.9)</td>
<td>7.4 (6.0, 14.8)</td>
<td>0.41</td>
</tr>
<tr>
<td>Pathologic Gleason sum (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>27.1</td>
<td>27.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>50.8</td>
<td>52.5</td>
<td></td>
</tr>
<tr>
<td>8+</td>
<td>22.0</td>
<td>20.3</td>
<td>Matched</td>
</tr>
<tr>
<td>Pathologic stage (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>13.6</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>T3a</td>
<td>44.1</td>
<td>44.1</td>
<td>Matched</td>
</tr>
<tr>
<td>T3b/N1</td>
<td>42.4</td>
<td>42.4</td>
<td></td>
</tr>
</tbody>
</table>

*Matched on age at surgery, race, pathologic stage, and Gleason sum in the prostatectomy specimen.
miRNA expression levels in prostate cancer recurrence cases and matched controls*, men who underwent prostatectomy for clinically localized disease, Johns Hopkins Hospital

<table>
<thead>
<tr>
<th>Relative normalized miRNA expression level**</th>
<th>Recurrence Cases</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>1.01 (0.80, 1.91)</td>
<td>1.54 (0.93, 2.79)</td>
<td>0.0149</td>
</tr>
<tr>
<td>miR-141</td>
<td>0.39 (0.21, 0.67)</td>
<td>0.81 (0.51, 1.49)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>miR-221</td>
<td>0.25 (0.14, 0.47)</td>
<td>0.46 (0.32, 0.78)</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

* Matched on age at surgery, race, pathologic stage, and Gleason sum in the prostatectomy specimen.

** Results do not take into account tissue block storage time effects. See Methods section.
Table 3
Association between miRNA expression levels and risk of prostate cancer recurrence after prostatectomy for clinically localized disease, Johns Hopkins Hospital

<table>
<thead>
<tr>
<th>Tertile of relative normalized miRNA expression level*</th>
<th>Number of cases/Number of controls</th>
<th>Matched** OR (95% CI)</th>
<th>Multivariable-adjusted† OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (lowest)</td>
<td>16/19</td>
<td>0.79 (0.34, 1.83)</td>
<td>0.81 (0.20, 3.29)</td>
</tr>
<tr>
<td>2</td>
<td>19/18</td>
<td>0.98 (0.41, 2.33)</td>
<td>1.08 (0.24, 4.74)</td>
</tr>
<tr>
<td>3 (highest)</td>
<td>24/22</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>P-trend</td>
<td></td>
<td>0.69</td>
<td>0.78</td>
</tr>
<tr>
<td>miR-141</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (lowest)</td>
<td>26/18</td>
<td>2.15 (0.86, 5.41)</td>
<td>2.12 (0.39, 11.6)</td>
</tr>
<tr>
<td>2</td>
<td>19/20</td>
<td>1.46 (0.58, 3.66)</td>
<td>0.69 (0.11, 4.50)</td>
</tr>
<tr>
<td>3 (highest)</td>
<td>14/21</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>P-trend</td>
<td></td>
<td>0.10</td>
<td>0.24</td>
</tr>
<tr>
<td>miR-221</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 (lowest)</td>
<td>28/18</td>
<td>3.21 (1.21, 8.53)</td>
<td>5.14 (0.99, 26.7)</td>
</tr>
<tr>
<td>2</td>
<td>21/19</td>
<td>2.63 (0.89, 7.78)</td>
<td>2.58 (0.38, 17.3)</td>
</tr>
<tr>
<td>3 (highest)</td>
<td>10/22</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>P-trend</td>
<td></td>
<td>0.02</td>
<td>0.05</td>
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</table>


** Matched on age at surgery, race, pathologic stage, and Gleason sum in the prostatectomy specimen.

† Further adjusted for year of surgery, presurgery serum PSA concentration, positive surgical margins, and residual difference in stage between cases and matched controls.
Supplementary Figure S1. Distribution of miRNA expression by calendar year of prostatectomy. Log transformed expression of (A) miR-21, (B) miR-221 and (C) miR-141 from cases and controls for year of surgery (categorized into early cases (1993-1994) or late cases (1995-2000)).