MicroRNA Signature Helps Distinguish Early from Late Biochemical Failure in Prostate Cancer

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PURPOSE: Prostate-specific antigen testing has led to overtreatment of prostate cancer (PCa). Only a small subset of PCa patients will have an aggressive disease that requires intensive therapy, and there is currently no biomarker to predict disease aggressiveness at the time of surgery. MicroRNAs (miRNAs) are reported to be involved in PCa pathogenesis.

METHODS: This study involved 105 participants. For the discovery phase, prostatectomy samples were dichotomized to high-risk (n=27, biochemical failure <36 months after prostatectomy) and low-risk groups (n=14, ≥36 months without biochemical failure). Expression of 754 mature miRNAs was compared between the 2 groups. Linear regression models were built to accurately predict biochemical failure risk. miRNA mimics were transfected into PCa model cell lines to test effects on proliferation and to deduce responding signaling pathways.

RESULTS: We identified 25 differentially expressed miRNAs between the biochemical failure risk groups. Based on the expression of 2–3 miRNAs, 3 logistic regression models were developed, each with a high positive predictive value. Candidate miRNAs and the best-performing model were also verified on an independent PCa set. miRNA-152, featured in the models, was further investigated by using cell line models and was shown to affect cell proliferation. Predicted interaction between miR-152 and Erbb3 (erythroblastic leukemia viral oncogene homolog 3) was experimentally validated in vitro.

CONCLUSIONS: miRNAs can help to predict biochemical failure risk at the time of prostatectomy.

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Only a small subset of prostate cancer (PCa)7 patients has an aggressive tumor that needs adjuvant treatment. For prognostic monitoring, the elevation of prostate-specific antigen (PSA) concentration in blood after prostatectomy (called biochemical failure, or biochemical relapse) remains the only available marker (1). PSA monitoring, however, cannot predict relapse at the time of surgery. Moreover, it has been shown that biochemical failure is not equal to clinical relapse (2). There is currently no biomarker that can accurately determine the risk of relapse at the time of prostatectomy.

Predicting relapse early can have important effects on patient outcome owing to closer follow-up and adjuvant therapy initiated for those with aggressive disease. Different approaches are used to estimate the risk of recurrence, e.g., the risk stratification model by D’Amico et al. (3); probability tables, such as the Partin table (4); or risk scores [e.g., the CAPRA (Cancer of the Prostate Risk Assessment) score] (5) and nomograms (6). However, independent validations have shown that these models still lack accuracy (7).

MicroRNAs (miRNAs) are short, single-stranded RNA molecules that regulate expression of their targets. They play key roles in different biological processes, such as stem cell maintenance, differentiation, organ development, and cancer pathogenesis (8) and metastasis (9, 10). miRNAs are differentially expressed...

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7 Nonstandard abbreviations: PCa, prostate cancer; PSA, prostate-specific antigen; miRNA, microRNA; Erbb3, v-erb-b2 erythroblastic leukemia viral oncogene homolog 3; FFPE, formalin-fixed paraffin-embedded; RT-qPCR, reverse-transcription quantitative PCR; DAVID, Database for Annotation, Visualization, and Integrated Discovery; Ct, cycle threshold; AR, androgen receptor; MAPK, mitogen-activated protein kinase; AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value; TGF-β, transforming growth factor-β; ECM, extracellular matrix; RPP, random pool of miRNA precursors; TCGA, The Cancer Genome Atlas database; NRG, neuregulin.

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in PCa (11–15) and have been shown to be involved in PCa pathogenesis, tumor progression, and metastasis (16). They also correlate with stage (14), perineural invasion (17), and androgen dependence (18).

In this study, we identified an miRNA signature that can distinguish between PCa patients with low-risk disease (no biochemical failure for ≥36 months after radical prostatectomy) from those who experienced an early biochemical failure (<36 months after radical prostatectomy). We developed logistic regression models to segregate the 2 groups. We also provide preliminary evidence showing that at least 1 of the miRNAs featured in the models, miR-152, affects PCa cell proliferation. Target prediction indicated that miR-152 can target v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (Erbb3). We experimentally validated the miRNA-target interactions in cell-line models.

Materials and Methods

PATIENT TISSUES AND RNA ISOLATION

Patients were approached for written informed consent before undergoing radical prostatectomy and agreed to donate surplus tissue samples for use in a research protocol approved by a research ethics board. Patient samples were obtained from the Charité School of Medicine, Berlin, Germany, and St Michael’s Hospital, Toronto, Canada. A total of 41 patients undergoing radical prostatectomy were included in the discovery phase; 27 suffered biochemical failure within 36 months of surgery (high-risk group), and 14 did not experience biochemical failure for at least 36 months following surgery (low-risk group) (clinical data are summarized in Table 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue11). The validation set consisted of 64 cases, including 35 patients from the high-risk group and 29 patients from the low-risk group.

Pure tumor areas from the primary Gleason grade were punched with use of the manual tissue arrayer (Beecher Instruments Tissue Microarray Technologies), creating cores 0.6 mm in diameter and 5 mm in length. Total RNA was isolated from 5–6 formalin-fixed paraffin-embedded (FFPE) cores with the FFPE miRNEasy kit (Life Technologies), following the manufacturer’s protocol. Total RNA from cultured cells was isolated with the miRNEasy kit (Life Technologies), following the manufacturer’s protocol.

Three milliliters urine and 0.6 mL serum were used for RNA extraction. Briefly, samples were mixed with 1.5 volumes QIAzol lysis reagent (Qiagen) and were cleaned with chloroform. RNA was precipitated from the aqueous phase overnight in 2 volumes of ice-cold 100% ethanol in the presence of glycogen. RNA concentration was measured with a NanoDrop 2000 (Thermo Scientific).

CELL LINE MODELS AND miRNA TRANSFECTION

PC3 and DU145 human PCa cell lines were purchased from American Type Culture Collection. Transfections were performed in 6-well plates with siPORT-NeoFX transfection agent (Life Technologies) as recommended by the manufacturer. Synthetic miRNA precursors were purchased from Life Technologies. We assessed cell proliferation by counting PCa cells 24 and 48 h after transient transfection, using a Vi-Cell automated viability counter (Beckman Coulter).

miRNA EXPRESSION SCREENING BY TaqMan LOW DENSITY ARRAY CARDS AND REVERSE-TRANScription QUANTITATIVE PCR ANALYSIS

We reverse transcribed 500 ng total RNA from each sample using a Megaplex Primer Pool Human Set v2.0 A + B (Life Technologies) with a TaqMan® miRNA reverse-transcription kit as suggested by the manufacturer. cDNA samples of individual patients were analyzed by a TaqMan® low-density array human microRNA card set v2.0 A + B. For validation, miRNAs were quantified by miRNA-specific TaqMan miRNA assays (Life Technologies) using 500 ng template total RNA. Reverse-transcription quantitative PCR (RT-qPCR) reactions were performed with TaqMan Fast Universal PCR mix (Life Technologies). Ectopic and endogenous miRNA concentrations were normalized against RNU48, and relative expression was calculated by the ΔΔ cycle threshold (Ct) method. Reverse transcription and qPCR reactions were performed on an ABI 7900HT machine.

Total cDNA was prepared with a high-capacity cDNA reverse transcription kit, and target miRNA was quantified by RT-qPCR. Expression was normalized against hypoxanthine phosphoribosyltransferase 1 (HPRT1)8 and ribosomal protein, large, P0 (RPLP0) endogenous expression. The following primers were used for this study: RPLP0, 5′-GGCGACCTGGAAGTC-CACT; RPLP0, 3′-CCATCAGGACACACACCCCTC; HPRT1, 5′-TTGCTGACCTGCTGGATTGAC; HPRT1, 3′-TCTCCCAAATATTTATCTG; ERBB2, 5′-TGATAGCACCAACCGCTCTC; ERBB2, 3′-GATTGGCATTGGACTCAAA; ERBB3, 5′-CTCTGGGGTTCTCTGCTGGAT; ERBB3, 3′-TGACCATACCATAGTT

8 Human genes: HPRT1, hypoxanthine phosphoribosyltransferase 1; RPLP0, ribosomal protein, large, P0, P0, phosphoinositide 3-kinase; AKT1, v-AKT murine thymoma viral oncogene homolog 1.
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CCTC; AR, 59-GACCAGATGGCTGTCATTCA; androgen receptor (AR), 39-GGAGCCATCCTTTTGTTA.

BIOINFORMATICS ANALYSIS
The TargetScan 6.2 miRNA prediction program was used for target prediction. Functional annotation was carried out with the Database for Annotation, Visualization, and Integrated Discovery (DAVID). Pathway analysis was performed using DAVID and the DIANA-miRPath TargetScan5.0 PicTar or DIANA-microT-3.0 Strict. Functional annotation was done through the Gene Ontology databases.

STATISTICAL ANALYSIS
A total of 371 miRNAs, or 49.2% of all investigated miRNAs, exhibited very low or undetectable expression levels in all samples (median Ct value of 35 or greater). To ensure accuracy and reproducibility, we focused subsequent analyses on a subset of 130 miRNAs (median Ct value 30 or lower). miRNA features were ranked by significance using 2 analytical techniques, the nonparametric ROC curve and the permutation t-test (using random permutations of participant status labels to derive empirical P values). Nonparametric Kruskal–Wallis tests were used to determine the significance of miRNAs among the biochemical failure categories. A subset of 130 miRNAs with higher expression levels (i.e., median Ct values <30.0) were selected for further analysis. Study participants were dichotomized into high-risk (i.e., biochemical failure within 3 years of surgery; n = 27) vs low-risk (i.e., biochemical failure more than 4 years after surgery; n = 14) categories. Binary logistic regression models were built to combine the segregation power of individual miRNAs.

miRNA expression values of the study set were clustered by SOTA (the Self-Organizing Tree Algorithm), available through MEV 4.0 (http://www.tm4.org/mev/).

To compare normalization methods, data were normalized against the Ct mean values of the small-nucleolar RNAs RNU48 and RNU44 or were subjected to geNorm software (qBasePLUS package) or NormFinder v0.953 (http://www.mdl.dk/publicationsnormfinder.htm). NormFinder determined miR-186, miR-30c, let-7d, and miR-191 as the best miRNA candidates for internal control.

PROTEIN AND RT-qPCR PROFILING ARRAYS
A Human Phospho-MAPK (mitogen-activated protein kinase) array kit was purchased from R&D. A total of 150 µg total protein was used for each membrane as directed by the manufacturer. Dot blots were quantified by VersaDoc™ Imaging Systems. Adjusted intensity values were obtained by use of Quantity One and Image Laboratory programs. An RT2 PCR profiler array for human phosphoinositide 3-kinase (PI3CA, also known as PI3K–v-AKT murine thymoma viral oncogene homolog (AKT1, also known as AKT) signaling was purchased from SABioscience.

Results
miRNAs ARE DIFFERENTIALLY EXPRESSED BETWEEN PCa PATIENTS WITH HIGH AND LOW RISK FOR BIOCHEMICAL FAILURE
Of 754 human miRNAs screened, 130 miRNAs showed expression Ct values of <30 and were used for subsequent analysis (see online Supplemental Table 2). Based on the permutation t-test rank, 25 miRNAs were significantly differentially expressed between the 2 risk groups. The ROC area under the curve (AUC) analysis identified 16 miRNAs statistically significant for differentiation between the low- and the high-risk biochemical failure groups (P < 0.05) (see online Supplemental Table 2). miRNAs identified by the 2 methods were highly overlapping. These miRNAs included multiple members of the miR-17–92 family (miR-19a, miR-19b, miR-20a, miR-20b) and the closely related miR-106a, miR-148a, miR-135a, miR-141, miR-19a, miR-19b, miR-374b, miR-26b, miR-20b, miR-374a, miR-29c, and miR-151–5p ranked among the top dysregulated miRNAs (Table 1). Correlations between miRNA expression and age, preoperative PSA concentration, and other clinical parameters were assessed and no significant correlations were found (see online Supplemental Table 2).

The expression of any single miRNA, however, was insufficient to accurately predict the biochemical failure risk category of that patient. Therefore, we conducted stepwise binary logistic regression analyses to construct models with high sensitivity and specificity. To avoid overfitting of models, we included only 2 or 3 miRNAs. Three models were developed and the predictive ability of these models was assessed by applying leave-one-out cross-validation methods. The first model is based on the expression of miR-331-3p and miR-152 (see online Supplemental Figs. 1A and 2). The ROC AUC was 0.931 (95% CI, 0.807–0.987). At a cutoff probability of 0.14, the model correctly classified 26/27 high-risk individuals (96.3%) and 8/14 low-risk individuals (57.1%); with a negative predictive value (NPV) of 8/9 (88.9%). At a cutoff probability of 0.86, the model correctly classified 21/27 high-risk individuals (77.8%) and 14/14 low-risk individuals (100%); with a positive predictive value (PPV) of 21/21 (100%) in our training set.

The second logistic regression model was based on the expression of 3 miRNAs: miR-331-3p, miR-152, and miR-135a (see online Supplemental Fig. 1B). In this model, the ROC AUC measured 0.929 (95% CI,
0.803–0.985), which was very similar to the performance of the first model. At a higher cutoff probability of 0.35, the model correctly classified 25/27 high-risk individuals (92.6%) and 11/14 low-risk individuals (78.6%), NPV 11/14 (84.6%), while at a cutoff probability of 0.9, the model correctly classified 22/27 high-risk individuals (81.5%) and 14/14 low-risk individuals (100%), PPV 22/22 (100%), as seen with the first model.

To improve the accuracy of detection of the low-risk group, we also developed a third model, based on the expression of miR-148a and miR-429 (see online Supplemental Fig. 1C and online Supplemental Fig. 2). In this case, the ROC AUC value was 0.788 (95% CI, 0.633–0.900). Setting the cutoff probability at 0.25, the model correctly classified 27/27 high-risk individuals (100%), PPV = 27/27 (100%). Meanwhile, at a cutoff probability of 0.898, the model correctly classified 11/27 high-risk individuals (40.7%) and 14/14 low-risk individuals (100%), PPV = 11/11 (100%). This model was inferior to the first model in overall accuracy; however, it could identify a subgroup of low-risk individuals with 100% NPV. The performance of the statistical models was also examined by using harmonized cutoff values, which did not result in substantial changes (see online Supplemental Box 1). In addition, different normalization strategies were applied and compared, including normalization against RNU44 and RNU48 mean Ct values, geNorm approach, and NormFinder. Results were comparable overall, with few exceptions (see online Supplemental Table 4).

### Table 1. Statistical significance of miRNAs differentially expressed between the low and high risk for biochemical failure groups.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Upregulated risk group</th>
<th>P (empirical t-test)*</th>
<th>ROC AUC value</th>
<th>Number of normalization methods showing significanceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-148a</td>
<td>Low-risk</td>
<td>0.006</td>
<td>0.78</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-1274b</td>
<td>Low-risk</td>
<td>0.009</td>
<td>0.75</td>
<td>NA</td>
</tr>
<tr>
<td>hsa-miR-141</td>
<td>Low-risk</td>
<td>0.012</td>
<td>0.74</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-135a</td>
<td>Low-risk</td>
<td>0.014</td>
<td>0.75</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-19a</td>
<td>Low-risk</td>
<td>0.015</td>
<td>0.74</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-19b</td>
<td>Low-risk</td>
<td>0.015</td>
<td>0.72</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-374b</td>
<td>Low-risk</td>
<td>0.02</td>
<td>0.74</td>
<td>NA</td>
</tr>
<tr>
<td>hsa-miR-26b</td>
<td>Low-risk</td>
<td>0.023</td>
<td>0.74</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-20b</td>
<td>Low-risk</td>
<td>0.025</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>hsa-miR-374a</td>
<td>Low-risk</td>
<td>0.025</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>hsa-miR-151-5p</td>
<td>Low-risk</td>
<td>NA</td>
<td>0.71</td>
<td>NA</td>
</tr>
<tr>
<td>hsa-miR-29c</td>
<td>Low-risk</td>
<td>NA</td>
<td>0.73</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-174b</td>
<td>Low-risk</td>
<td>NA</td>
<td>NA</td>
<td>5</td>
</tr>
<tr>
<td>hsa-miR-196b</td>
<td>Low-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-26a</td>
<td>Low-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-331-3p</td>
<td>High-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-193a</td>
<td>High-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-365</td>
<td>High-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-125a</td>
<td>High-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>hsa-miR-125b</td>
<td>High-risk</td>
<td>NA</td>
<td>NA</td>
<td>3</td>
</tr>
</tbody>
</table>

* P values are given for the top 10 differentially expressed miRNAs.

b miRNAs significant with ≥3 normalization methods are indicated.

NA, not assessed.
d miR-1274b is likely a derivative of tRNAh153.

 VALIDATION OF miR-331-3P AND miR-152 DIFFERENTIAL EXPRESSION

We validated our results using miRNA-specific probe-based RT-qPCR for 2 miRNAs that are featured in the logistic regression models: miR-331-3p and miR-152. miR-331-3p showed significant downregulation (P = 0.009) in the low-risk biochemical failure group. Also, in agreement with the initial screening, miR-152 expression was significantly lower in the high-risk group.
To further confirm our results, we validated the differential expression of miR-331-3p and miR-152 on a second independent set of PCa cases (n = 64). We confirmed significant downregulation of miR-331-3p in the low-risk group and significant downregulation of miR-152 in the high risk for biochemical failure patients (P = 0.01), as expected (Table 2).

To further validate our findings, we tested the performance of the first logistic regression model on the independent patient set. A univariate logistic model containing miR-152 alone was predictive for biochemical failure risk (P = 0.043). Inclusion of miR-331-3p to miR-152 provided significant improvement to the predictive ability of the model (P = 0.004). At a cutoff probability of 0.30, the model correctly classified 33/35 high-risk individuals (94.3%) and 8/29 low-risk individuals (27.6%). At the harmonized cutoff probability of 0.28, the model correctly classified 33/35 high-risk individuals (94.3%) and 6/29 low-risk individuals (20.7%).

To further validate differential miRNA expression between the risk groups, we quantified miR-29c expression across the validation set patients. miR-29c expression correlated with PSA-free survival. Moreover, we examined miR-29c expression in metastatic PCa lesions. miR-29c expression was significantly lower in metastatic tumors, confirming its downregulation in more aggressive lesions (see online Supplemental Fig. 3).

Combination of the study set and the validation set was used to assess possible dependence of miRNA expression on age. miR-152 and miR-331-3p did not correlate with age (see online Supplemental Fig. 4). Two other significant microRNAs were tested in normal prostate tissue, urine, and serum and were shown to be independent of age (see online Supplemental Figs. 5 and 6).

**miR-152 AFFECTS PROLIFERATIVE ABILITY OF PCa CELLS**

Two of the 3 models featured miR-152 and miR-331-3p. Moreover, miR-148a, used in the third model, shares the same seed region (CAGUGCA) with miR-152 and is therefore predicted to have overlapping targets. miR-148a and miR-152 are reported to regulate cell proliferation, migration, and invasion in cancer (19) and to promote differentiation (20). miR-331-3p has been shown to induce cell-cycle arrest in gastric cancer and to play a role in castration-resistant PCa (21).

We examined the effect of miR-152 on PCa behavior through gain-of-function experiments in cell line models. DU145 cells transfected with miR-152 exhibited a 57% decrease in cell proliferation, and PC3 cells showed a 35% reduction of proliferation compared to control (Fig. 1). Our results show a negative effect of miR-152 on cell proliferation and are in agreement with the decreased miR-152 expression seen in the high-risk

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**Table 2. Validation of miR-152 and miR-331-3p differential expression on the study set and an independent set of prostate cancer cases.**

<table>
<thead>
<tr>
<th>miR</th>
<th>Validation on the study set</th>
<th>Validation on an independent set</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean expression fold change (low- vs high-risk group)</td>
<td>P</td>
</tr>
<tr>
<td>miR-152</td>
<td>2.2</td>
<td>0.012</td>
</tr>
<tr>
<td>miR-331-3p</td>
<td>0.51</td>
<td>0.009</td>
</tr>
</tbody>
</table>

**Fig. 1. miR-152 affects prostate cancer cell proliferation in vitro.**

(A) The DU145 PCa cell line was treated with the siPORT transfection agent (sip) or miR-152 (152) or miR-331-3p (331-3p). miR-152 overexpression resulted in significant reduction of cell proliferation in 48 hours. (B) The PC3 PCa cell line was treated with the siPORT transfection agent or transfected with miR-152 or miR-331-3p. miR-152 significantly decreased cell proliferation, whereas miR-331-3p had no effect.
biochemical failure group. These results are also consistent with recent reports showing that increased proliferative activity of PCa cells is a prognostic marker for recurrent PCa after radical prostatectomy (22). Independent studies have demonstrated that miR-148a, which is highly similar to miR-152, inhibits cell growth, migration, and invasion of PC3 cells (23).

Transfection of miR-331-3p did not lead to significant changes in the proliferative ability of DU145 and PC3 cells (Fig. 1). This miRNA was upregulated in the high-risk biochemical failure group (Table 1) and was therefore not expected to reduce the rate of cell proliferation.

**DIFFERENTIALLY EXPRESSED miRNAs CAN TARGET PCa PATHOGENESIS-RELATED GENES**

To understand the mechanisms by which miR-152 may contribute to PCa progression, we first conducted a functional clustering analysis and pathway analysis on their predicted targets. The most significant predicted pathways included the transforming growth factor-β (TGF-β) signaling, focal adhesion, extracellular matrix (ECM)-receptor interaction, and Erbb signaling pathways (Fig. 2 and online Supplemental Table 5). The same results were obtained when using the top 5 or top 10 most significantly different miRNAs between the high and low risk for biochemical failure groups. As a control, we performed the same analysis using a random miRNA set and a set of miRNAs that are not significantly dysregulated between high- and low-risk groups. These pathways were not recognized as significant categories.

**VALIDATION OF miR-152–ERBB3 INTERACTION AND THEIR DOWNSTREAM TARGETED PATHWAYS**

To experimentally confirm miR-152–Erbb3 interaction, we overexpressed miR-152 in PC3 and LNCaP cells. The Erbb3 mRNA concentration remained unchanged in PC3 cells treated with siPORT transfection agent or transfected with a random pool of miRNA precursors (RPP). However, Erbb3 mRNA expression dropped by 44% upon miR-152 overexpression (Fig. 3A). We observed a similar decrease of Erbb3 mRNA expression levels when LNCaP cells were transfected with miR-152. AR mRNA expression also decreased upon miR-152 transfection, showing a possible indirect effect of miR-152 (Fig. 3B).
To validate our findings in vivo, we compared Erbb3 expression in patients’ samples between the 2 biochemical failure risk groups using the validation set samples from St Michael’s Hospital (Toronto). We dichotomized patients to Erbb3-expressing and Erbb3-nonexpressing groups (Ct cutoff value 39). Only 1 of 8 (12.5%) of high-risk patients had no measurable Erbb3 expression; whereas 8/27 (30%) of low-risk patients failed to show measurable Erbb3 expression, indicating increased Erbb3 concentrations and an inverse correlation with miR-152 in the high-risk category.

To further validate our findings, we took advantage of the publicly available The Cancer Genome Atlas (TCGA) database. Erbb3 expression was significantly higher in PCa patients who experienced early biochemical failure (<24 months, n = 56) compared with patients with late biochemical failure (≥24 months, n = 39, P = 0.009).

The Erbb3 tyrosine kinase is activated by neu-regulins (NRGs) or other Erbb and non-Erbb kinases and it connects to the downstream PI3K/AKT/MAPK mitogenic pathway. Erbb3 can also activate AR independently of the PI3K/AKT pathway by stabilizing AR protein in complex with Erbb2 (24), and secreted Erbb3 promotes bone metastasis (25). The activated Erbb/PI3K/Akt/nuclear factor-κB pathways were suggested to predict biochemical recurrence of PCa. Moreover, Erbb3 is reported to be responsible for therapy resistance in different cancers and is a promising therapeutic target (26). To examine a possible downstream effect of miR-152 on PI3K and MAPK pathways, LNCaP cells were transfected with miR-152. PI3K pathway activity was monitored by RT-qPCR array while MAPK pathway activity was followed by protein profiling array. Preliminary results indicated that miR-152 overexpression interfered with the protein concentrations of p-AKT1 and p-AKT2 (see online Supplemental Fig. 7 and online Supplemental Table 6). Changes in the PI3K pathway members were less evident, with no molecules reaching a 2-fold difference. It should be noted, however, that miRNA effects on a specific pathway can be the sum of an “miRNA network” rather than an individual miRNA effect.

We also used the TCGA database to compare expression of the downstream PI3K and MAPK pathway members. Our pilot analysis showed differential expression of several members of these pathways for aggressive tumors (high risk for biochemical failure, and/or high Gleason score) compared to less aggressive ones (see online Supplemental Table 7).

In summary, these data indicate that miR-152 is a likely regulator of the Erbb3 and may act through downstream PI3K and MAPK pathways; however, further investigation is required to establish its additional regulatory roles.

Discussion

In current practice, the combination of PSA concentration, Gleason score, and clinical stage provides the best tool for prediction of PCa survival. Biochemical failure can indicate relapse only after it has occurred; therefore, biomarkers that predict PCa aggressiveness are urgently needed.
Exome sequencing for genetic alterations has confirmed frequent somatic mutations related to PCA. mRNA profiling and epigenetic markers are candidate indicators for biochemical recurrence and prognosis after surgery, and noninvasive markers such as serum-based biomarkers and circulating tumor cells are of emerging interest. miRNAs possess attractive characteristics that make them ideal biomarkers. Their relative stability in formalin-fixed tissues allows extraction and quantification from clinical samples, and a large number of miRNAs can be quantified in parallel by a multiplex RT-qPCR. Finally, recent studies have shown that miRNAs are present in a stable form in body fluids like serum and urine and that these fluids can be used for noninvasive testing (10).

Among the 130 miRNAs that showed high expression in our present study, 18 have already been linked to PCA relapse (11–13, 27–30). Our results are in agreement with earlier reports that showed that miR-26b, miR-30a-3p, and miR-26a correlate with biochemical failure (12).

Based on our bioinformatics and experimental analyses, the PCA-related Erbb/PI3K/Akt/MAPK pathway is a potentially important target of miR-152. Erbb signaling was reported as a major steroid-independent activator of AR. Erbb3 overexpression has recently been linked with resistance to Erbb1 inhibitors in lung cancer (31), and mutation of Erbb4, a closely related receptor tyrosine kinase, inhibits the formation of drug-resistant colonies in PCA cell lines (32), making miR-152 interesting not only as a prognostic marker but also as a therapeutic tool. miRNA therapy offers the benefit of simultaneous downregulation of multiple genes and cancer-promoting signaling pathways, and recently miRNA-122 inhibitor has successfully completed the first phase II clinical trial for hepatitis C virus (Santaris Pharma A/S).

In summary, we established 3 statistical models that provide useful information to predict biochemical failure at the time of prostatectomy. These logistic regression models appear to be useful in identifying patients at a high risk for biochemical failure. Our study evaluated a large number of potential miRNA markers on a relatively small-sized training set (41 individuals). This design is inherently predisposed to biased marker selection and can lead to markers that are highly specific for the given patient population but perform less well on an independent or a larger set of patients. Further evaluation of potential miRNA markers on a larger patient population is required to determine their accuracy as markers of PCA progression. Our experimental data support that miR-152 targets Erbb3 and has an indirect effect on AR expression at the mRNA level; its exact downstream regulatory effect, however, needs further confirmation. The ability to quantify miRNA expression from formalin-fixed tissues and body fluids makes this approach potentially useful in determining prognosis in the clinic. Exploration of the molecular events during PCA progression will likely lead to the identification of new and more effective therapeutic targets.

References

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