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Circulating microRNAs predict biochemical recurrence in prostate cancer patients

L A Selth^{*,1}, S L Townley¹, A G Bert², P D Stricker³, P D Sutherland⁴, L G Horvath^{5,6}, G J Goodall^{2,7}, L M Butler¹ and W D Tilley¹ on behalf of the Australian Prostate Cancer BioResource

¹Dame Roma Mitchell Cancer Research Laboratories, Adelaide Prostate Cancer Research Centre, University of Adelaide and Hanson Institute, PO Box 14, Rundle Mall, Adelaide, South Australia 5000, Australia; ²Division of Human Immunology, Centre for Cancer Biology, SA Pathology, Adelaide, South Australia 5000, Australia; ³Department of Urology, St Vincent's Clinic, Darlinghurst, New South Wales 2010, Australia; ⁴Urology Unit, Surgical Specialties Service, Royal Adelaide Hospital, Adelaide, South Australia 5000, Australia; ⁵Department of Medical Oncology, Sydney Cancer Centre, Royal Prince Alfred Hospital, Missenden Road, Camperdown, New South Wales 2050, Australia; ⁶Cancer Research Program, The Kinghorn Cancer Centre/Garvan Institute of Medical Research, Darlinghurst, New South Wales 2010, Australia and ⁷Discipline of Medicine, The University of Adelaide, Adelaide, South Australia 5005, Australia

Background: Circulating microRNAs (miRNAs) are emerging as promising biomarkers for prostate cancer. Here, we investigated the potential of these molecules to assist in prognosis and treatment decision-making.

Methods: MicroRNAs in the serum of patients who had experienced rapid biochemical recurrence (BCR) ($n = 8$) or no recurrence ($n = 8$) following radical prostatectomy (RP) were profiled using high-throughput qRT-PCR. Recurrence-associated miRNAs were subsequently quantitated by qRT-PCR in a validation cohort comprised of 70 patients with Gleason 7 cancers treated by RP, 31 of whom had undergone disease progression following surgery. The expression of recurrence-associated miRNAs was also examined in tumour tissue cohorts.

Results: Three miRNAs – *miR-141*, *miR-146b-3p* and *miR-194* – were elevated in patients who subsequently experienced BCR in the screening study. *miR-146b-3p* and *miR-194* were also associated with disease progression in the validation cohort, as determined by log-rank tests and Cox proportional hazards regression. Multivariate analysis revealed that *miR-146b-3p* possessed prognostic information beyond standard clinicopathological parameters. Analysis of tissue cohorts revealed that *miR-194* was robustly expressed in the prostate, elevated in metastases, and its expression in primary tumours was associated with a poor prognosis.

Conclusion: Our study suggests that circulating miRNAs, measured at the time of RP, could be combined with current prognostic tools to predict future disease progression in men with intermediate risk prostate cancers.

Prostate cancer is a major cause of cancer-related death (Jemal *et al*, 2011). However, disease course is highly variable and difficult to predict: a significant proportion of cases are indolent and do not require curative treatment, whereas others are characterised by high metastatic potential and require aggressive, early clinical intervention. Unfortunately, current clinicopathological models, which incorporate parameters such as Gleason score, tumour stage

and serum prostate-specific antigen (PSA) concentration, are unable to accurately differentiate between indolent and life-threatening cancers at the time of diagnosis. Subsequently, there is considerable overtreatment of indolent disease, accompanied by high morbidity. The issue of overtreatment has been further compounded by PSA testing (Andriole *et al*, 2009; Schroder *et al*, 2009; Chou *et al*, 2011). More accurate prognostic

*Correspondence: Dr LA Selth; E-mail: luke.selth@adelaide.edu.au

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tools would have a major impact on the clinical management of prostate cancer.

MicroRNAs (miRNAs) are small non-protein-coding RNA molecules that regulate gene expression by binding to complementary sequences in target mRNAs and mediating translational repression and/or mRNA degradation. A recent estimate suggests that ~60% of all protein-coding genes are directly targeted by miRNAs (Friedman *et al*, 2009). It is therefore unsurprising that miRNAs have demonstrated functions in a diverse array of processes, including development, cell behaviour, stress responses and survival (Bushati and Cohen, 2007).

In addition to their roles in normal physiology, miRNAs are frequently dysregulated in cancer (Croce, 2009). This paradigm suggests that miRNAs could be clinically useful cancer biomarkers, a concept recently validated by the launch of miRNA-based diagnostic tests for thyroid and pancreatic cancer by Asuragen. The potential of miRNAs for biomarker applications was further reinforced by recent research showing that cancer-specific miRNAs are detectable in cell-free blood fluids such as serum and plasma (Lawrie *et al*, 2008; Mitchell *et al*, 2008). Since those seminal studies, circulating miRNAs have emerged as promising biomarkers for various malignancies (Weiland *et al*, 2012). In relation to prostate cancer, most studies to date have focused on identifying circulating miRNAs that can assist in diagnosis or the stratification of metastatic from localised disease (Kelly *et al*, 2012; Selth *et al*, 2012). While a subset of these molecules have been linked to prognostic clinical variables (Brase *et al*, 2011; Selth *et al*, 2011), the utility of circulating miRNAs to predict disease course at the time of diagnosis remains inconclusive. In this study, we report on the ability of circulating miRNAs to predict biochemical recurrence (BCR) in men with localised prostate cancer treated by radical prostatectomy (RP).

MATERIALS AND METHODS

Human serum samples. The Australian Prostate Cancer BioResource (APCB) collected biological samples and clinical data from men undergoing RP at the Royal Adelaide Hospital (RAH) and St Vincent's Hospital (SVH), Sydney. All men gave written consent for sample and ongoing data collection, and practices and procedures were approved by the RAH and SVH Human Research Ethics Committees. Blood was collected according to the APCB's standard operating procedures, which have been reviewed within Australia and by the International Society of Biological and Environmental Repositories. Samples were collected in 8-ml Serum Separator Gel Vacuette tubes (Greiner BioOne, Frickenhausen, Germany; catalogue number 455078), allowed to clot for ~30 min at room temperature and centrifuged at room temperature for 10 min at 2500 g. Serum was aliquoted into Nunc cryovials (ThermoFisher Scientific, San Jose, CA, USA), snap frozen in liquid nitrogen and stored at -80°C . Patients were followed post-operatively by their surgeons on a monthly basis until satisfactory urinary continence was obtained and then at 3-month intervals until the end of the first year, at 6-monthly intervals to 5 years and yearly thereafter. Relapse was defined by the following criteria: biochemical disease progression with a serum PSA concentration of 0.2 ng ml^{-1} increasing over a 3-month period.

RNA extraction. Total RNA was extracted from serum essentially as described previously (Selth *et al*, 2011), with some minor modifications. Before extraction, serum was centrifuged at 3000 g for 5 min at 4°C and transferred to a new 1.5 ml tube. A serum to Qiazol ratio of 1:5 was used to denature the samples. For the samples profiled by high-throughput qRT-PCR, miRNAs were extracted from 500 μl serum spiked with 25 fmol synthetic *cel-miR-39* (Integrated DNA Technologies, Coralville, IA, USA) and eluted in

35 μl RNase-free water. For the validation samples, miRNAs were extracted from 200 μl serum spiked with 25 fmol synthetic *cel-miR-39* and eluted in 100 μl RNase-free water. Additionally, 1 μg of yeast transfer RNA (Roche, Mannheim, Germany) was added to the smaller validation serum samples as a carrier.

TaqMan Low-Density arrays. Serum samples from two disparate patient groups – men who experienced rapid BCR following RP and men who were progression free at the time of last follow-up – were profiled using TaqMan Low-Density Array (TLDA) Human MicroRNA A + B Cards Set v3.0 (Applied Biosystems, Foster City, CA, USA), as per the manufacturer's instructions. We used 3 μl of serum miRNA as input for the Megaplex reverse transcription (RT) reactions. After RT and pre-amplification, quantitative PCR was carried out on an Applied Biosystems 7900HT. Cycle threshold values were calculated using SDS 2.4 software (Applied Biosystems) with manual baseline (3–15) and threshold (0.2) settings.

Undetected miRNAs (average quantification cycle value (Cq) > 35 in either the BCR or no BCR groups) were removed from the analysis. Normalisation of the data was as follows: (1) the A plates and B plates were normalised to each other using the Cq values for *U6* snRNA, which is amplified on both plates; (2) all data were then scaled to the spiked-in *cel-miR-39* to correct for differences in extraction efficiency, (3) finally, the mean Cq value of all miRNAs with a Cq value of < 30 for each array was determined and used to normalise the arrays. This global normalisation strategy improves upon methods based on internal reference genes (Mestdagh *et al*, 2009; Deo *et al*, 2011). Raw and normalised data have been deposited in Gene Expression Omnibus (accession number GSE47125).

Quantitative RT-PCR analysis of circulating miRNAs in validation cohort. To validate miRNAs identified by TLDA profiling, TaqMan Human miRNA assays (Applied Biosystems) were used to measure the levels of *miR-141*, *miR-146b-3p*, *miR-194* and *miR-375* (assay numbers 000463, 002361, 000493 and 000564, respectively) in 70 independent patient serum samples enriched for men who had experienced recurrence post-RP. Spiked-in *cel-miR-39* (assay number 000200) was used for normalisation purposes. TaqMan MicroRNA Reverse Transcription Kits (Applied Biosystems) were used to synthesise cDNA from 4 μl of total serum RNA (in 10 μl reactions). The miRNA-specific stem-loop primers for *miR-141*, *miR-146b-3p*, *miR-194*, *miR-375* and *cel-miR-39* were pooled, dried down in a speed-vac, resuspended in RNase-free water and used at a final concentration of $0.3125\times$, as described in the Applied Biosystems Protocol for Creating Custom RT and Preamplification Pools using TaqMan MicroRNA Assays. One microlitre of the RT product was used in 10 μl pre-amplification reactions containing $0.5\times$ concentration of *miR-141*, *miR-146b-3p*, *miR-194* and *miR-375* TaqMan MicroRNA assays, as described previously (Selth *et al*, 2011). The pre-amplification product was then diluted 10-fold in RNase-free water and 2 μl used in real-time PCRs containing $1\times$ concentrations of individual TaqMan MicroRNA assays and 5 μl of TaqMan Universal Master Mix, No AmpErase UNG (Applied Biosystems). *Cel-miR-39* was quantified by adding 2 μl of 10-fold diluted cDNA (not pre-amplified) in real-time PCRs as described above. Cq values of endogenous miRNAs were normalised to *cel-miR-39* Cq values, which ranged from 19.60 to 22.45 (mean 20.73, standard deviation 0.59). In 10 samples, *miR-146b-3p* was not detected (Cq > 40); for statistical analyses, the Cq values for these samples were set to the maximum Cq across all samples (Madhavan *et al*, 2012).

The efficiency and specificity of the assays were confirmed by using synthetic oligonucleotides (Sigma-Aldrich, St Louis, MO, USA) corresponding to mature miRNAs *miR-141*, *miR-146b-3p*, *miR-194*, *miR-375* and *cel-miR-39* (sequences obtained from miRBase, release 19). Single oligonucleotide (1 fmol) or an oligonucleotide pool (1 fmol of each) was added to single plex

(i.e., containing single miRNA stem-loop RT primers) or multiplexed (i.e., containing all five miRNA stem-loop RT primers) RT reactions. Reverse transcription products were diluted over four orders of magnitude; the serial dilutions were used in qPCRs containing single Taqman assays to generate standard curves and to determine efficiency values for each assay. Two microlitres of each RT product (1:10 000 diluted) was also used as template in either single plex or multiplexed pre-amplification reactions. Pre-amplified cDNA was diluted over four orders of magnitude and used to generate standard curves and to define qPCR efficiency. Finally, assay specificity was assessed by determining the relative detection of the assay-specific synthetic miRNA vs the four non-specific synthetic miRNAs in qPCRs following multiplexed RT/pre-amplification. Cross-reactivity values were calculated based on the C_q difference between assay-specific and non-specific synthetic miRNAs and expressed as percent relative detection.

Analysis of circulating miRNAs in human tumour samples.

Serum miRNA markers of prostate cancer recurrence were analysed in two tumour data sets (Taylor *et al*, 2010; Martens-Uzunova *et al*, 2011). The Memorial Sloan-Kettering Cancer Center data set contains miRNA expression data from 99 primary prostate cancers, 28 non-malignant tissues and 14 metastases. The Erasmus Medical Center data set contains miRNA expression data from 50 primary tumours obtained by RP, 22 primary tumours obtained by Transurethral Resection of the Prostate (TURP), 12 lymph-node metastases and 15 normal prostate tissues. Both tissue cohorts were profiled using Agilent microRNA V2 arrays. Raw data were downloaded from GEO (accession number GSE21036) and ArrayExpress (accession number E-TABM-794) and processed using AgiMicroRNA (Lopez-Romero, 2011). Clinical data for the Erasmus Medical Center cohort were kindly provided by Drs E Martens-Uzunova and G Jenster.

Statistical analyses. Biochemical recurrence-free survival was measured from the date of RP to relapse or the date of last follow-up. Differences in serum miRNA levels between the BCR and progression-free groups, as defined by TLDA profiling, were assessed using *t* tests. The association between serum miRNA levels and BCR in the validation cohort was assessed using Kaplan–Meier survival curves and univariate and multivariate Cox proportional hazards regression. The capacity of serum miRNAs to distinguish between men who experienced recurrence and men who remained disease free was evaluated by ROC curve analysis. Predicted values from logistic regression models were used to generate ROC curves from a combination of variables (i.e., serum miRNAs plus clinical variables). Kruskal–Wallis one-way ANOVA tests were employed to compare miRNA levels among normal prostate tissue, primary tumours and metastatic samples. All statistical analyses were done using GraphPad Prism (version 5; GraphPad Software, San Diego, CA, USA) and MedCalc (version 12; MedCalc Software, Mariakerke, Belgium).

RESULTS

Circulating miRNAs associated with BCR following RP. Serum samples from 16 patients (Table 1) representing two disparate groups – men who experienced rapid BCR following RP and men with a mean of 53.4 months follow-up post-RP but no evidence of BCR – were profiled using TLDA. The two groups were otherwise closely matched in terms of clinicopathological parameters such as Gleason score, primary Gleason grade and staging to increase the likelihood of identifying miRNAs that may complement existing prognostic tools. All samples were collected from patients treated at RAH, South Australia. One sample from the BCR group was disregarded following TLDA profiling because the clinical follow-up data could not be verified.

Table 1. Clinical details of patient samples used in screening study

Variable	Total (n = 16)	BCR (n = 8)	No BCR (n = 8)	P-value ^a
Age, mean ± s.e.m. (years)	66.1 ± 1.1	65.8 ± 1.4	66.4 ± 1.8	0.857
Time to BCR, mean ± s.e.m. (months)	—	18.9 ± 4.4	—	—
Follow-up, mean ± s.e.m. (months)	54.4 ± 2.8	55.4 ± 5.7	53.4 ± 2.4	0.687
Pre-operative PSA (ng ml ⁻¹)				0.282
< 10	11	4	7	
≥ 10	5	4	1	
Primary Gleason grade				0.467
3	7	3	4	
4	8	5	3	
5	1	0	1	
Gleason score				1.000
7	12	6	6	
8	0	0	0	
9	4	2	2	
Pathologic stage				1.000
pT2	6	3	3	
pT3	10	5	5	

Abbreviations: BCR = biochemical recurrence; PSA = prostate-specific antigen.
^aMann–Whitney *U*-test or Fisher's exact test *P*-value.

After data filtering and normalisation (see Materials and methods), 217 miRNAs were detectable in circulation (Figure 1A). Of these, only *miR-141*, *miR-146b-3p* and *miR-194* were differentially expressed between the BCR and no BCR groups (*P* < 0.05, no adjustment for multiple testing) (Figure 1B). *MiR-141* has been identified as a marker of high-risk and metastatic prostate cancer in previous studies (Mitchell *et al*, 2008; Brase *et al*, 2011; Selth *et al*, 2011; Bryant *et al*, 2012), suggesting that the TLDA data were robust and our experimental approach was well founded. Other miRNAs previously associated with high-risk and metastatic disease, including *miR-375* and *miR-200b* (Brase *et al*, 2011; Selth *et al*, 2011; Bryant *et al*, 2012), were elevated in men who relapsed but the differences were not statistically significant (Figure 1C).

Recent reports suggest that peripheral blood cells can be a major source of circulating miRNAs (Pritchard *et al*, 2012). In the absence of blood cell counts or haemoglobin concentrations for our samples, we investigated the levels of miRNAs known to be highly expressed in white (WBC) and red (RBC) blood cells: *miR-150* and *miR-223* are markers of lymphoid and myeloid WBCs, respectively (Pritchard *et al*, 2012), while both *miR-16* and *miR-451* are present at high concentrations in RBCs and can be used to assess haemolysis during serum preparation (Kirschner *et al*, 2011). The C_q values for these miRNAs are shown in Figure 2A. The expression level and variation in range of C_q values for the individual miRNAs (*miR-150*, 2.4; *miR-223*, 2.6; *miR-16*, 3.2; *miR-451*, 4.4) was comparable to that observed in previous studies (Kirschner *et al*, 2011; Pritchard *et al*, 2012), suggesting that the levels of miRNAs determined by TLDA qRT-PCR were unlikely to be significantly impacted by contaminating cellular miRNAs. Furthermore, none of the cellular miRNAs were differentially expressed between the BCR and no BCR groups (Figure 2B).

Circulating miR-146b-3p and miR-194 are predictive of disease recurrence following RP. To validate the findings from the screening study, we collected a larger, independent set of patient

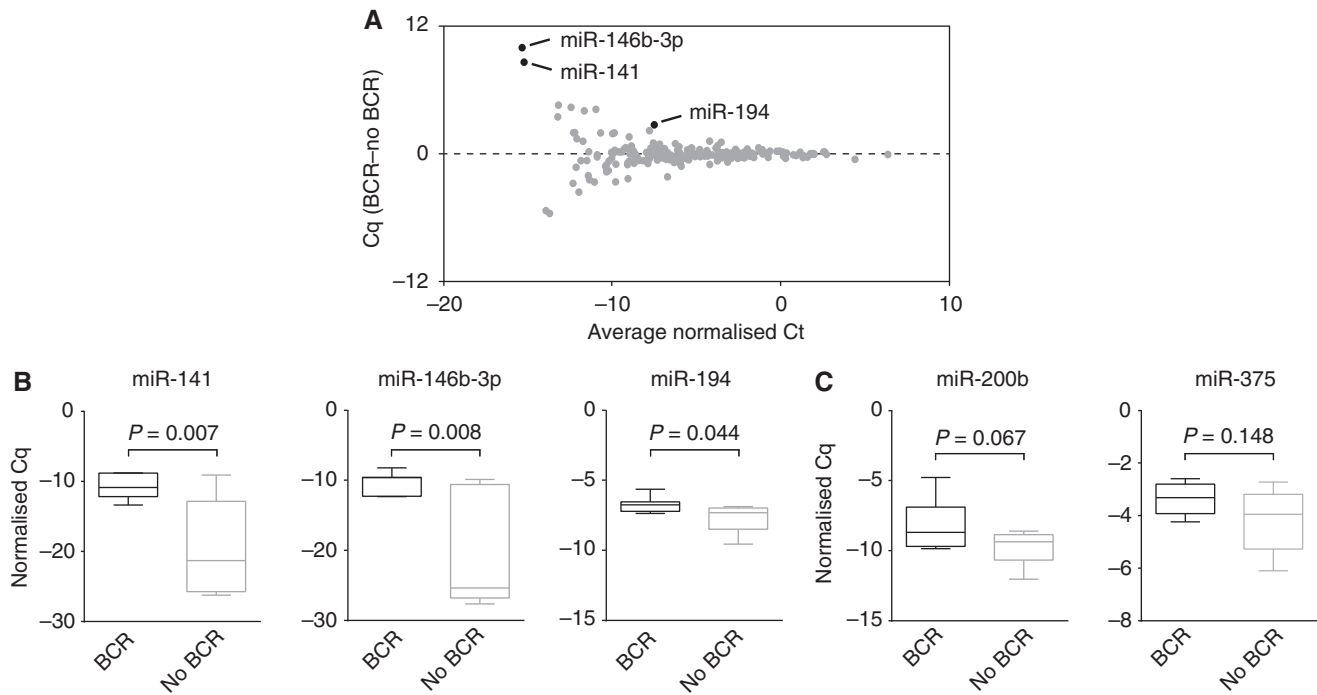


Figure 1. Circulating miRNA markers of biochemical recurrence (BCR). **(A)** Bland–Altman mean-difference plot of 217 circulating miRNAs detected in a screening cohort of prostate cancer patients. MiRNAs found to be significantly differentially expressed between the BCR ($n=8$) and no BCR ($n=8$) groups are shown in black. **(B)** Relative levels of *miR-141*, *miR-146b-3p* and *miR-194* in the serum of men who experienced BCR and men who did not. Statistically significant differences were assessed using a *t*-test; *P*-values are shown. The lines within boxes represent the median, the horizontal borders of each box represent the 25th and 75th percentile, and the limits of the vertical lines represent the maximum and minimum Cq. **(C)** Relative levels of *miR-200b* and *miR-375* in the serum of men who experienced BCR and men who did not, graphed as described in **(B)**.

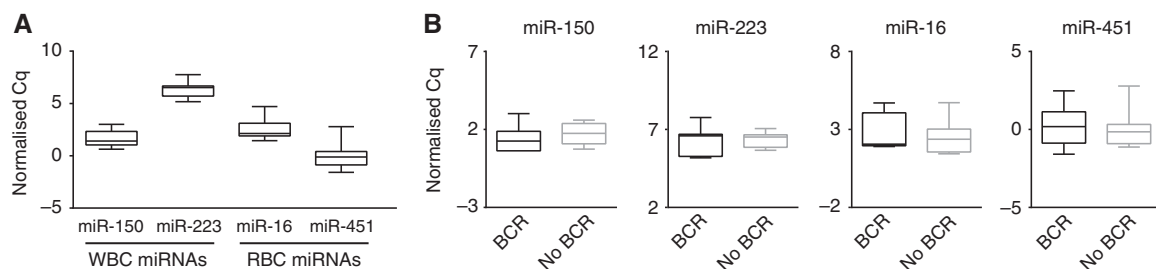


Figure 2. Variation in serum levels of miRNAs expressed at high levels in blood cells. **(A)** Levels of *miR-150* and *miR-223* (markers of white blood cells (WBC)) and *miR-16* and *miR-451* (markers of red blood cells (RBC)) in the serum samples used in the screening study. **(B)** Relative levels of blood cell miRNAs in the serum of men who experienced biochemical recurrence (BCR; $n=8$) and men who did not ($n=8$). None of the miRNAs were significantly different between the two groups. The lines within boxes represent the median, the horizontal borders of each box represent the 25th and 75th percentile, and the limits of the vertical lines represent the maximum and minimum Cq.

samples from a different site (SVH, New South Wales) to those analysed in the screening study. This validation cohort was comprised of 70 patients and enriched for men who had experienced early BCR following the RP ($n=31$; Table 2). For men who progressed, the median time from RP to recurrence was 24.7 months. All of the cancers had a Gleason score of 7 (3 + 4 or 4 + 3); as such, the patients represent an intermediate risk group that would benefit from additional markers of disease behaviour (D'Amico *et al*, 1998). The three miRNAs found to be significantly elevated in patients who subsequently relapsed in the screening study (*miR-141*, *miR-146b-3p* and *miR-194*) were quantitated in serum samples from these patients using qRT-PCR. We also measured *miR-375*, since it has been previously correlated with adverse risk factors and it was elevated in the BCR group, albeit non-significantly, in our screening study. Levels of the candidate

BCR-associated miRNAs were normalised to spiked-in *cel-miR-39* only, since there is no consensus on suitable endogenous small RNAs in circulation for such a purpose (Kroh *et al*, 2010).

Before quantitation of circulating miRNAs in the validation cohort, we assessed whether multiplexing the RT and pre-amplification reactions affected the efficiency and/or specificity of the *miR-141*, *miR-146b-3p*, *miR-194*, *miR-375* and *cel-miR-39* assays. All of the assays performed similarly when used in combination (multiplexed) or individually (Figure 3A). Moreover, qPCR efficiency values of the assays following multiplexed RT/pre-amplification ranged from 93.8% to 104.1%, within the commonly employed functional cutoff of 90–110% (Figure 3A). To evaluate the specificity of the assays, we utilised synthetic mature miRNA molecules as templates in multiplexed RT/pre-amplification reactions followed by qPCR with individual assays. Only the

Table 2. Clinical details of patient samples used in validation study

Variable	Total (n = 70)	BCR (n = 31)	No BCR (n = 39)	P-value ^a
Age, mean ± s.e.m. (years)	62.2 ± 0.8	62.6 ± 1.1	61.9 ± 1.1	0.648
Follow-up, mean ± s.e.m. (months)	64.2 ± 1.4	65.1 ± 2.0	63.4 ± 2.0	0.872
Pre-operative PSA (ng ml ⁻¹)				0.091
< 10	53	20	33	
≥ 10	17	11	6	
Gleason score				0.053
3 + 4	37	12	25	
4 + 3	33	19	14	
Pathologic stage				0.114
pT2	33	11	22	
pT3	36	19	17	
pT4	1	1	0	
Surgical margins				0.275
R0	51	20	31	
R1	18	10	8	
Rx	1	1	0	
Seminal vesicle invasion				0.018
Negative	62	24	38	
Positive	8	7	1	

Abbreviations: BCR = biochemical recurrence; PSA = prostate-specific antigen.
^aMann-Whitney U-test or Fisher's exact test P-value.

miR-141 and *miR-146b-3p* assays exhibited any detectable cross-reactivity, but relative detection of the non-specific miRNA was 4–5 orders of magnitude less than the specific miRNA (Figure 3B). Collectively, these results indicated that quantitation of circulating *miR-141*, *miR-146b-3p*, *miR-194* and *miR-375* using a multiplexed Taqman assay was a robust approach.

Kaplan–Meier (KM) analysis was used to test for an association between the circulating miRNAs and BCR. For each miRNA, patients were dichotomised into two groups based on median levels. Higher circulating levels of *miR-146b-3p* and *miR-194* were associated with an increased risk of BCR but *miR-141* and *miR-375* were not (Figure 4). The prognostic capacity of the circulating miRNAs was further assessed using Cox proportional hazard regression. Univariate analysis demonstrated that high circulating levels of *miR-146b-3p* were associated with a reduced recurrence-free interval, as was pre-operative serum PSA concentration, Gleason grade and seminal vesicle invasion (SVI) (Table 3). A combined value representing the mean normalised Cq of *miR-146b-3p* and *miR-194* possessed greater prognostic value ($P = 0.004$, hazard ratio = 1.29 (95% confidence interval (CI) = 1.07–1.57)). In multivariate analysis, *miR-146b-3p*, pre-operative PSA and SVI were significant predictors of time to BCR (Table 3), indicating that this circulating miRNA provides prognostic information independent of standard clinicopathological parameters.

The capacity of circulating *miR-146b-3p* and *miR-194* to distinguish between men who subsequently recurred and men who remained disease free was further evaluated by ROC curve analysis. Circulating *miR-146b-3p* (area under the curve (AUC) = 0.62, $P = 0.095$), *miR-194* (AUC = 0.65, $P = 0.029$), pre-operative PSA (AUC = 0.69, $P = 0.003$) and SVI (AUC = 0.60, $P = 0.013$) all possessed similar discriminatory power. Adding the miRNA variables to SVI significantly improved its discriminatory power (AUC of SVI + miRNAs = 0.75; SVI alone = 0.60; P -value of difference = 0.007), whereas the AUC of PSA combined with the

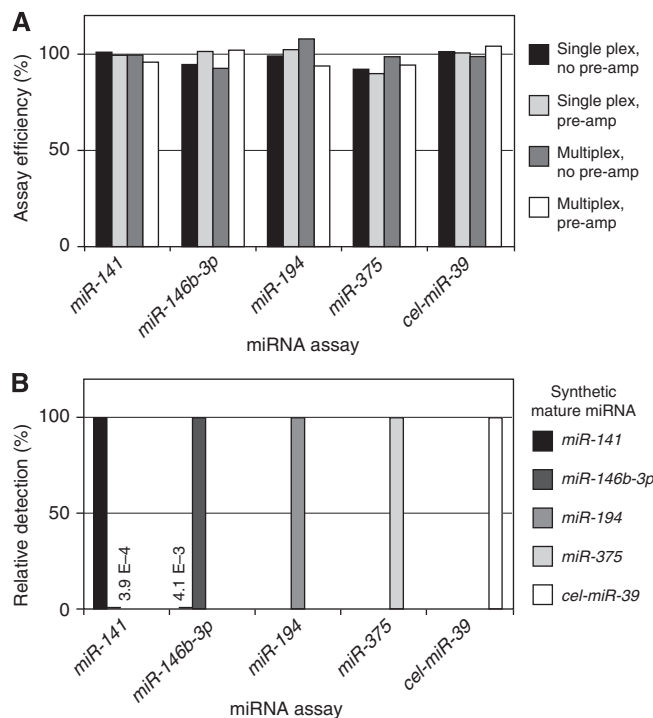


Figure 3. Efficiency and specificity of Taqman miRNA assays. (A) PCR efficiencies for each assay in either single plex or multiplexed reactions, with and without pre-amplification. (B) Specificity of the miRNA assays. Cross-reactivity between the assays was calculated based on the Cq difference between assay-specific (set to 100%) and non-specific synthetic miRNA targets. Values for the two cases of detectable cross-reactivity are shown on the graph.

miRNAs was only marginally increased compared with PSA alone (PSA + miRNAs = 0.74; PSA alone = 0.69; $P = 0.297$) (Figure 5).

We also evaluated the correlation between circulating miRNAs and clinicopathological factors (Table 4). Neither *miR-146b-3p* nor *miR-194* was associated with primary Gleason grade, pathologic stage or SVI. Increased *miR-194* was positively correlated with age, but *miR-146b-3p* was not. Interestingly, *miR-146b-3p* was negatively correlated with margin status. Both miRNAs showed a trend towards positive correlation with pre-operative serum PSA levels, but this did not reach statistical significance.

Expression of circulating miRNAs in prostate cancer. Given their association with outcome, we hypothesised that circulating *miR-146b-3p* and *miR-194* were derived from cancer cells. As a preliminary effort to test this hypothesis, the expression of these miRNAs was evaluated in tumour samples. Two clinical cohorts were evaluated, one from Memorial Sloan-Kettering Cancer Center (MSKCC cohort) and the other from the Erasmus Medical Center (Erasmus cohort). In both of the cohorts, *miR-146b-3p* expression in normal and malignant tissues was very low (not detectable in 38 out of 99 (Erasmus) and 80 out of 141 (MSKCC) samples), precluding further analysis. By contrast, *miR-194* was robustly expressed in normal and malignant prostate cells and was found to be significantly higher in both lymph-node metastases (Erasmus) and a mixed population of metastases (MSKCC) (Figure 6A). Kaplan–Meier survival analysis revealed that high *miR-194* was a poor prognostic indicator in the Erasmus cohort, but this association did not reach statistical significance (Figure 6B, left). When the MSKCC cohort was dichotomised by the median *miR-194* expression, no association with BCR was observed (data not shown). However, the highest quartile of *miR-194* in the MSKCC cohort had a significantly poorer prognosis than the lower quartile

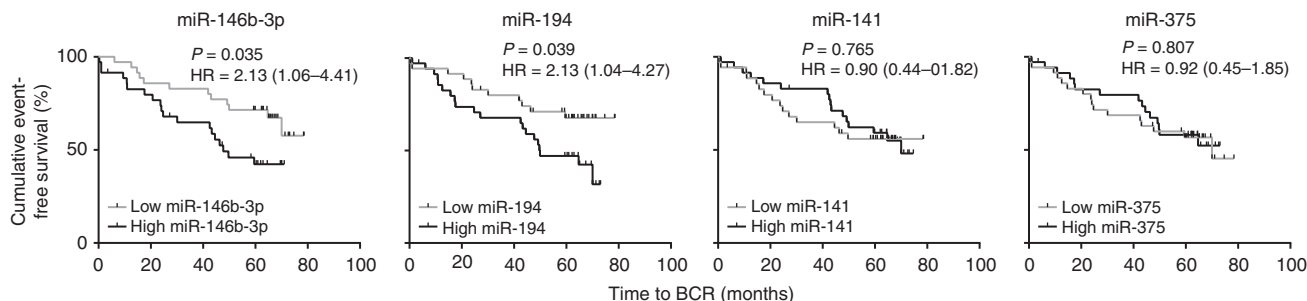


Figure 4. Kaplan-Meier analysis in the validation set (n = 70) showing estimated biochemical recurrence (BCR)-free probability in patients with high (above median) or low (below median) levels of circulating miRNAs. Curves were compared using a log-rank test. P-values and hazard ratios (HRs; 95% confidence interval in parentheses) are shown.

Table 3. Univariate and multivariate Cox proportional hazard analysis of circulating miRNAs and clinicopathological parameters in relation to recurrence-free interval after radical prostatectomy

Variable ^a	Univariate		Multivariate			
	HR (95% CI) ^b	P-value	Inclusion	P-value	Stepwise ^c	P-value
miR-141	0.97 (0.75–1.26)	0.829	0.91 (0.68–1.21)	0.520		
miR-146b-3p	1.13 (1.01–1.26)	0.020	1.16 (1.03–1.31)	0.017	1.14 (1.02–1.27)	0.019
miR-194	1.13 (0.97–1.31)	0.112	1.08 (0.91–1.28)	0.399		
miR-375	0.88 (0.55–1.43)	0.613	0.83 (0.46–1.49)	0.526		
Pre-operative PSA	1.14 (1.06–1.24)	0.001	1.10 (1.00–1.20)	0.045	1.11 (1.02–1.20)	0.013
Gleason score (3 + 4 vs 4 + 3)	2.29 (1.11–4.71)	0.023	1.56 (0.60–4.10)	0.366		
Pathologic stage (pT2 vs pT3)	1.86 (0.90–3.88)	0.090	1.43 (0.53–3.85)	0.481		
Surgical margins (R0 vs R1)	1.45 (0.68–3.09)	0.352	1.00 (0.34–2.95)	0.998		
SVI (negative vs positive)	3.86 (1.64–9.05)	0.007	2.59 (0.91–7.41)	0.077	3.50 (1.47–8.29)	0.005

Abbreviations: CI = confidence interval; HR = hazard ratio; SVI = seminal vesicle invasion.

^amiRNAs and pre-operative PSA are continuous variables.

^bFor miRNAs, the hazard ratio refers to an increase of 1 normalised Ct value.

^cReduced model following stepwise backward or forward elimination.

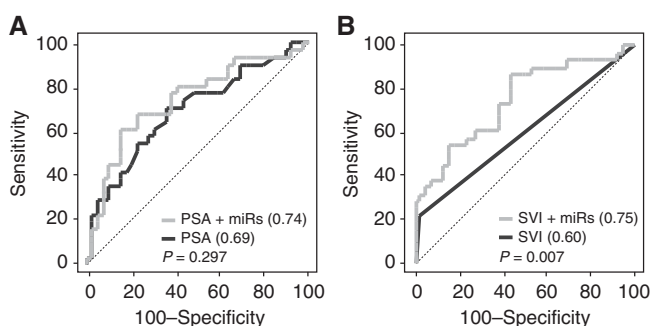


Figure 5. ROC curve analysis in the validation set (n = 70) comparing the ability of clinical parameters alone or in combination with circulating miRNAs to identify men who experience biochemical recurrence. (A) Comparison of pre-operative PSA alone (dark grey) or combined with circulating miR-146b-3p and miR-194 (light grey). (B) Comparison of seminal vesicle invasion (SVI) alone (dark grey) or combined with circulating miR-146b-3p and miR-194 (light grey). Area under the curve (AUC) is shown in brackets. The Delong method was used to compare the AUCs; P-values are shown.

(Figure 6B, right). Univariate Cox proportional hazard regression revealed that high miR-194 was significantly correlated with time to BCR in the MSKCC cohort (P = 0.033, hazard ratio = 3.33 (95% CI = 1.12–9.87)) and with time to metastasis in the Erasmus cohort (P = 0.026, hazard ratio = 3.75 (95% CI = 1.28–10.97)).

DISCUSSION

Biomarkers that provide accurate prognostic information for men with prostate cancer are urgently required. Here, we demonstrate for the first time that circulating miR-146b-3p and miR-194, measured immediately before RP, can be used to predict disease progression in patients of intermediate risk. This finding was achieved by employing a set of samples with comprehensive long-term clinical outcome data. In addition, circulating miR-146b-3p and miR-194 were only weakly correlated with other clinical variables, suggesting that they provide new prognostic information. Therefore, these markers have the potential to improve the management of intermediate risk localised prostate cancers, which would be of significant clinical utility.

Table 4. Correlation between serum miR-146b-3p and miR-194 levels and clinicopathological parameters

Variable	miR-146b-3p ^a	P-value ^b	miR-194 ^a	P-value ^b
Age, years				
< 65 (n = 40)	- 7.98	0.143	3.77	0.004
≥ 65 (n = 30)	- 8.84		5.32	
Pre-operative PSA (ng ml⁻¹)				
< 10 (n = 53)	- 8.77	0.095	4.30	0.073
≥ 10 (n = 17)	- 7.03		5.18	
Gleason score				
3 + 4 (n = 37)	- 8.65	0.923	4.35	0.285
4 + 3 (n = 33)	- 8.11		4.90	
Pathologic stage				
pT2 (n = 33)	- 7.96	0.411	4.88	0.671
pT3/pT4 (n = 37)	- 8.77		4.58	
Surgical margins				
R0 (n = 51)	- 7.96	0.014	4.58	0.389
R1 (n = 18)	- 9.97		5.49	
Seminal vesicle invasion				
Negative (n = 62)	- 8.57	0.419	4.76	0.906
Positive (n = 8)	- 7.55		5.08	

Abbreviation: PSA = prostate-specific antigen.
^aMedian normalised Ct.
^bMann-Whitney test.

While *miR-146b-3p* has not been directly linked to prostate cancer, there is accumulating evidence for a role of the *miR-146* family in this malignancy. In an early miRNA profiling study, *miR-146a-5p* was shown to be commonly overexpressed in solid tumours, including prostate cancer (Volinia *et al*, 2006). More recently, it was reported that the expression of both *miR-146a-5p* and *miR-146b-5p* was reduced in a cell line model of castration-resistant prostate cancer compared with androgen-dependent prostate cancer cells (Lin *et al*, 2008) and *miR-146b-5p* was decreased in metastatic vs non-metastatic xenografts (Watahiki *et al*, 2011). How the loss of *miR-146* during prostate cancer progression fits with our observation that serum *miR-146b-3p* is a marker of disease recurrence is currently unclear. Interestingly, the 5p and 3p forms of *miR-146b* have converse associations with recurrence of non-small cell lung cancer (Patnaik *et al*, 2009, 2011), providing a precedent for distinct functions of the *miR-146b* pairs in cancer progression. Despite its association with disease behaviour, *miR-146b-3p* does not appear to be expressed at high levels in normal or malignant prostate tissue. We envision at least three explanations for this apparent discrepancy. First, *miR-146b-3p* could be efficiently released from cancer cells into circulation such that it does not accumulate in the tumour. Preferential cellular export of certain miRNAs has been reported previously (for review, see Selth *et al*, 2012). Indeed, *miR-1* and *miR-133a* are increased in serum in response to acute myocardial infarction but the levels of both are reduced in the infarcted myocardial tissue (Kuwabara *et al*, 2011), indicating that large discrepancies between the levels of miRNA in circulation compared with their tissue of origin are possible. Second, serum *miR-146b-3p* may originate from circulating tumour cells (CTCs) and/or micrometastases that

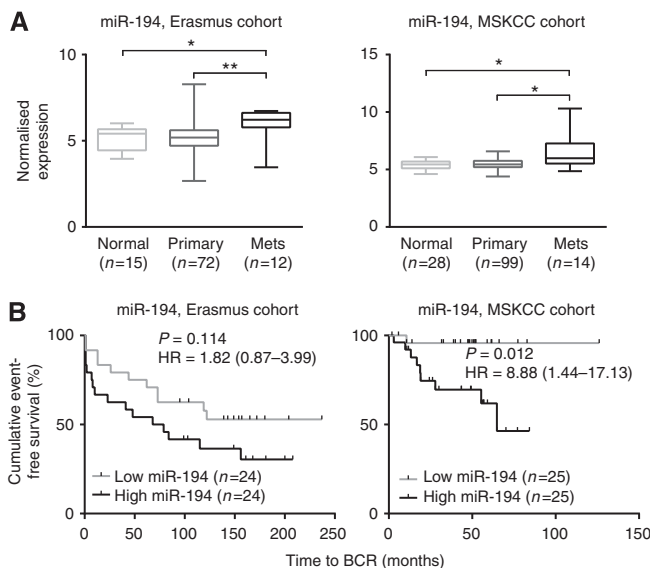


Figure 6. Expression and prognostic value of *miR-194* in prostate tumours. (A) Relative expression of *miR-194* in normal prostate tissue, primary prostate tumours (Primary) and metastases (Met). Two different cohorts, Erasmus and MSKCC were analysed. LN, lymph nodes. Statistically significant differences were assessed using a Kruskal-Wallis one-way ANOVA (* $P < 0.05$; ** $P < 0.01$). **(B)** Kaplan-Meier curves showing estimated biochemical recurrence (BCR)-free probability in patients with high (above median) or low (below median) intra-tumoural expression of *miR-194* in the two cohorts. Curves were compared using a log-rank test: P -values and hazard ratios (HR; 95% confidence interval in parentheses) are shown. BCR, biochemical relapse.

have not been detected at the time of treatment. Given its association with disease recurrence, we favour this explanation. Finally, we cannot rule out the possibility that circulating *miR-146b-3p* does not originate from malignant prostate cells but rather from systemic changes in response to the disease, such as inflammatory responses.

In support of our discovery that circulating *miR-194* is associated with disease progression, Tong *et al* (2009) reported that this miRNA was elevated in tumours from men who experienced rapid BCR (within 2 years of RP) compared with men who did not experience BCR ≥ 10 years after surgery. Our analysis of two large, published data sets further demonstrated that the intra-tumoural expression of *miR-194* was associated with poor outcome measures, including BCR and metastasis, although further analysis in larger cohorts is required to make more definitive conclusions. Collectively, these observations suggest that *miR-194* may be expressed in high-risk tumours and released into circulation during carcinogenesis. Interestingly, *miR-194* was recently found to be elevated in the serum of a mouse model of diffuse-type gastric cancer, suggesting that it may be a more general circulating marker of malignancy (Rotkrue *et al*, 2013).

Of all the circulating miRNAs previously associated with prostate cancer, *miR-141* and *miR-375* have been the most commonly identified (Mitchell *et al*, 2008; Brase *et al*, 2011; Gonzales *et al*, 2011; Selth *et al*, 2011; Bryant *et al*, 2012; Nguyen *et al*, 2012). These earlier studies demonstrated associations between *miR-141/miR-375* and clinicopathological parameters (Gleason score, pathologic stage and lymph-node status), metastatic disease and treatment response. However, while both of these

miRNAs were elevated in men who experienced BCR in the screening cohort (note that for *miR-375*, this was a non-significant change), neither were correlated with BCR in the larger validation cohort. Therefore, the potential of these circulating miRNAs as clinically useful markers for prostate cancer remains to be conclusively proven. With this in mind, it is worth noting that studies of circulating miRNAs to date are highlighted by conflicting data and outcomes, which likely arise from heterogeneity in terms of objectives and methodology. Improving the consistency and standardisation of these factors is of critical importance for future work.

A role in epithelial–mesenchymal plasticity is an emerging link between many of the circulating miRNAs associated with prostate cancer. *MiR-141* and *miR-200b* are members of the *miR-200* family, which target the *Zeb1/2* transcription factors and thereby repress epithelial–mesenchymal transition (EMT) (Bracken *et al*, 2009). *MiR-429* is another *miR-200* family member that was significantly elevated in the BCR group of our screening cohort, although it was filtered from further analysis because of low expression (average Cq = 41.3 in the screening samples). *MiR-194* can also inhibit EMT in models of liver (Meng *et al*, 2010) and endometrial cancer (Dong *et al*, 2011) and may directly target the mesenchymal factor N-cadherin (Song *et al*, 2011). Additionally, a recent study found that *miR-375* could partially reverse EMT in MCF-7 breast cancer cells (Ward *et al*, 2012). It is currently unclear why these ‘epithelial’ miRNAs are elevated in the circulation of men with metastatic prostate cancer (i.e., *miR-141*, *miR-200b* and *miR-375*; Mitchell *et al*, 2008; Brase *et al*, 2011; Selth *et al*, 2011; Bryant *et al*, 2012) or localised prostate cancer with a poor prognosis (*miR-194*, this study). One possibility is that they are indicative of CTCs and/or metastases that have undergone or are undergoing a mesenchymal–epithelial transition (MET), a process likely to be important for efficient metastatic colonisation (Gao *et al*, 2012). Supporting this concept, Madhavan *et al* (2012) recently demonstrated that circulating *miR-200* family members and *miR-375* are surrogate markers for CTCs in breast cancer and correlated with disease progression and overall survival. The circulating levels of *miR-200* family members have also been associated with a poor outcome in gastric and endometrial cancer (Valladares-Ayerbes *et al*, 2012; Torres *et al*, 2013). Collectively, these findings suggest that disruption of MET to prevent metastatic colonisation may be an effective therapeutic strategy.

Even with a pre-amplification step, some serum prostate cancer-associated miRNAs reported previously and in this study (e.g., *miR-141* and *miR-146b-3p*) are at the limit of detection by Taqman qRT-PCR. Moreover, others that are potentially useful markers of BCR (e.g., *miR-429*) were discarded from our analyses because of low concentration. We believe that emerging technologies, such as NanoString, digital PCR and miRNA-specific nanopores (Wang *et al*, 2011), will increase the accuracy of miRNA detection from cell-free fractions of body fluids and thereby enhance the utility of these molecules as disease-specific markers.

A limitation of this study is the validation cohort, which was small, limited to Gleason score 7 patients, and enriched for men who experienced early biochemical disease progression. Moreover, although BCR has become the *de facto* measure of the effectiveness of RP (Nielsen and Partin, 2007), it does not always translate into clinical progression. Indeed, there have been conflicting reports regarding the utility of BCR as a predictor of metastasis and cancer-specific survival (Agarwal *et al*, 2008; Boorjian *et al*, 2011). As such, the results should be interpreted with caution and with the caveat that additional studies might uncover circulating miRNAs that add to or outperform the markers identified here, particularly in relation to prognosing systemic progression and/or prostate cancer death.

Despite these limitations, it is worth reinforcing the potential utility of circulating miRNAs such as *miR-146b-3p* and *miR-194*

that may assist in prognosis of intermediate risk patients, a group that engenders considerable clinical uncertainty. We intend to validate our findings in a larger, prospectively collected cohort of such patients and assess whether these miRNAs add to pre-operative nomograms and thereby have the potential to reduce rates of overtreatment. In this respect, it is important to note that whereas pre-operative clinical parameters, such as Gleason score, can be affected by undersampling (Haas *et al*, 2007) and/or molecular heterogeneity of multifocal disease (Barry *et al*, 2007), circulating miRNAs are likely to provide an overall snapshot of changes occurring in response to tumour growth. Moreover, circulating miRNAs can be measured non-invasively and repeatedly, which may assist in treatment decision-making. We also believe that circulating miRNAs are likely to have potential as prognostic markers in men treated by active surveillance or in men with high-risk localised cancers, two further applications that will be assessed in future work.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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