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A circulating miRNA assay as a first-line test for prostate cancer screening

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Background: Prostate cancer (PCa) screening currently relies on prostate-specific antigen (PSA) testing and digital rectal examination. However, recent large-scale studies have questioned the long-term efficacy of these tests, and biomarkers that accurately identify PCa are needed.

Methods: We analysed the levels of circulating microRNAs (miRNAs) in patients with elevated PSA who were diagnosed with either localised PCa ($n=36$) or benign prostatic hyperplasia (BPH, $n=31$) upon biopsy. Real-time RT–PCR with Taqman probes was used to measure plasma levels of miRNAs. To circumvent problems associated with circulating miRNA quantitation, we computed the expression ratios of upregulated and downregulated miRNAs.

Results: The miR-106a/miR-130b and miR-106a/miR-223 ratios were significantly different between the biopsy-positive and BPH groups ($P<0.0001$), and yielded statistical power values that were >0.99. Both miRNA ratios were highly sensitive and more specific than PSA in discriminating localised PCa from BPH. Receiver operating characteristic curve analysis revealed area under curve values of 0.81 (miR-106a/miR-130b) and 0.77 (miR-106a/miR-223).

Conclusions: Testing for circulating miR-106a/miR-130b and miR-106a/miR-223 ratios may reduce the costs and morbidity of unnecessary biopsies and is feasible for large-scale screening, as it requires measuring only three miRNAs.

Prostate cancer (PCa) is the most frequent cancer in males in Europe and North America. Digital rectal examination (DRE) and serum prostate-specific antigen (PSA) monitoring followed by serial biopsy and histological analysis is the cornerstone of PCa screening/diagnosis. However, although very high PSA values indicate a high probability of cancer detection by biopsy, lower values poorly discriminate PCa from inflammatory processes and benign hyperplasia (Ploussard *et al*, 2013; Hayes and Barry, 2014). On the basis of the results of two large epidemiological studies showing a very small or no effect of PSA testing on PCa mortality (Andriole *et al*, 2012; Schroder *et al*, 2012) and the negative consequences of PSA screening, which include overdiagnosis, overtreatment and treatment complications, the US Preventive Services Task Force recommended against the use of PSA testing for PCa screening (Aly *et al*, 2015; Johnson *et al*, 2015).

These considerations have engendered an interest in the identification of novel markers of PCa with improved specificity compared with PSA. Circulating microRNAs (miRNAs; Bartel, 2004; Waltering *et al*, 2011; Kim and Kim, 2013) may represent excellent biomarkers because of their ease of detection, stability in biological fluids and minimal invasiveness of the test. Different studies identified specific miRNA signatures associated with PCa (Mahn *et al*, 2011; Zhang *et al*, 2011; Chen *et al*, 2012; Kelly *et al*, 2015; Mihelich *et al*, 2015), although a clear consensus has not yet

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emerged. Discrepant findings may in part reflect differences in pre-analytical sample processing and data normalisation/analysis.

In the present study, we explored the efficacy of circulating miRNA as biomarkers for PCa. To circumvent problems linked to data normalisation, we calculated ratios of upregulated and downregulated miRNAs. Results indicated that the miR-106a/miR-130b and miR-106a/miR-223 ratios are highly sensitive and more specific than PSA in discriminating localised PCa from benign prostatic hyperplasia (BPH).

MATERIALS AND METHODS

Patients. A total of 67 patients were recruited; 36 were positive for PCa upon biopsy (BIO+) and 31 were negative (BIO−). BIO+ and BIO− patients were comparable in terms of mean age (65.2 and 66.7 years, respectively) and PSA (8.5 and 8.4 ng ml^{-1}, respectively). All the BIO+ patients had localised PCa, and all of the BIO− patients were diagnosed with BPH. Additional clinical characteristics of the patients are described in the Supplementary Materials and Methods and Supplementary Table S1.

Quantitative RT–PCR. RNA was extracted from plasma as described in the Supplementary Materials and Methods. Five microlitres of total RNA were used for first-strand cDNA synthesis in a 15-μl reaction volume, using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Thermo Fisher Scientific, Foster City, CA, USA). cDNAs (2.5 μl) were amplified for 45 cycles using TaqMan miRNA primers and probes (Thermo Fisher Scientific) and LightCycler 480 PCR Master Mix (Roche Diagnostics GmbH, Mannheim, Germany). No-RT and no-template negative controls were included. The amplification reactions were performed in a LightCycler 480 II (Roche). Signals were quantified using the second derivative maximum method (Software Version 1.5, Roche). All amplification curves were carefully examined, and products with poor curves were discarded. Ct values <40 were obtained for $>99\%$ of the reaction samples.

Data normalisation. As candidate normalisers we tested spiked-in cel-miR-39 as well as human miR-24, which exhibited low variability in our data set (data not shown) and was used as a normaliser in other studies (Bianchi *et al*, 2011).

Relative expression of target miRNAs was calculated as $\Delta Ct_{miR} = Ct_{miR} - Ct_{normalizer}$. Differential expression of each miRNA was then evaluated as $\Delta\Delta Ct_{miR} = \Delta Ct_{miR}(\text{patient}) - \Delta Ct_{miR}(\text{control})$ i.e., by subtracting the mean ΔCt value for the BIO− group from the ΔCt of each BIO+ patient.

To circumvent biases linked to the choice of an appropriate reference, we also used the miRNA ratio approach (Boeri *et al*, 2011). The Ct value of each miRNA was converted to the corresponding expression level (2^{-Ct}). Twenty-one different miRNA ratios were calculated as $2^{-Ct_{miR-x}}/2^{-Ct_{miR-y}} = 2^{-(Ct_{miR-x}-Ct_{miR-y})} = 2^{-\Delta Ct}$.

Statistical analysis. The two-tailed Wilcoxon rank-sum test was used to identify miRNAs significantly different between the BIO+ and BIO− groups, whereas the one-tailed Wilcoxon rank-sum test was used for miRNA ratios. *Post hoc* power analysis was performed using a nonparametric resampling technique (10 000 replications). A univariate logistic regression model was built to evaluate the ability of each miRNA ratio to predict the BIO+ outcome. The two most significant miRNA ratios were also used in a bivariate logistic regression model. Odds ratios and 95% confidence intervals were estimated for the fitted logistic regression models. Receiver operating characteristic (ROC) curves were plotted and the area under the ROC curve (AUC) was estimated to compare the two most significant miRNA ratios. Optimal thresholds were determined using the Youden's J statistic. Sensitivity, specificity, negative predictive value, positive predictive value and accuracy were determined. Statistical analyses were performed in the R environment, using a customised code and the pROC package for ROC curve analysis.

RESULTS

Circulating miRNA in PCa and BPH patients. We examined 36 patients with localised PCa confirmed by serial biopsy (BIO+) and 31 individuals diagnosed with BPH (BIO−). Clinical assessment of the patients included age, PSA, DRE, Gleason scoring and T stage (Supplementary Table S1). Twelve miRNAs were selected among those reported to be involved in PCa (Supplementary Table S2). To facilitate their application to large-scale testing, we focused on miRNAs that could be detected with a single round of qRT–PCR (i.e., without a pre-amplification step).

Several studies underscored the critical importance of data normalisation when measuring circulating miRNAs. Pilot tests with the cel-miR-39 spike-in reference showed a wide variability in Ct values (Supplementary Figure S1), possibly due to differences in plasma RNAase levels, as previously reported (Mahn *et al*, 2011). We thus normalised each miRNA using as internal reference miR-24, which was relatively constant in our samples and was used as a normaliser in other studies (Bianchi *et al*, 2011). Results showed that miR-106a, miR-20a, miR-223 and miR-21 were significantly different between BIO+ and BIO− individuals (Figure 1).

miRNA ratios distinguish PCa (BIO+) from BPH (BIO−) patients. To overcome the bias linked to data normalisation using a calibrator RNA, we next calculated the ratios between the expression values of miRNA pairs listed in Supplementary Table S3. Fourteen ratios were found to be significantly different between the BIO+ and BIO− groups with $P<0.05$. We focused on the miR-106a/miR-130b and miR-106a/miR-223 ratios (Figure 2A), which ranked highest in terms of statistical significance (*P*-values were <0.0001) and showed a statistical power >0.99 (see Supplementary Table S3 for details). As shown in Figure 1, miR-106a was upregulated in BIO+ samples, whereas miR-130b and miR-223 were downregulated.

These miRNA ratios were next evaluated by univariate logistic regression analysis. Resulting areas under ROC curves (AUC) were 0.81 and 0.77 for miR-106a/miR-130b and miR-106a/miR-223, respectively (Figure 2B); bivariate logistic regression analysis using both miRNA ratios as predictors showed an AUC of 0.84 (Figure 2B). All these values were considerably higher than the AUC of 0.56 obtained for PSA, a value consistent with other studies (Guzel *et al*, 2015). Odds ratios are reported in Supplementary Tables S4 and S5.

Determining threshold values for miRNA ratios. Statistical analysis indicated optimal $2^{-\Delta Ct}$ threshold values of 10.413 for miR-106a/miR-130b and 0.176 for miR-106a/miR-223. To facilitate application in clinical practice, results of the bivariate logistic regression model were transformed into a miRNA score = 0.1855 × (miR-106a/miR-130b) + 2.3162 × (miR-106a/miR-223), where the miRNA ratios indicate $2^{-\Delta Ct}$ values. The optimal threshold for this miRNA score was 2.142. With these cutoff values, specificity and sensitivity were 0.87 and 0.72 (miR-106a/130b), 0.65 and 0.81 (miR-106a/223), and 0.81 and 0.83 (miRNA score), respectively.

A diagnostic flowchart for PCa screening. On the basis of these findings, we propose a flowchart for the selection of patients to be subjected to biopsy (Figure 2C). Patients with PSA >4 ng ml^{-1} (right-hand portion of the chart) would first be tested for miR-106a/miR-130b; patients with a $2^{-\Delta Ct}>10.413$ would be eligible for biopsy, whereas patients below this threshold would be tested for the miR-106a/miR-223 ratio. Patients with a miR-106a/miR-223 $2^{-\Delta Ct}>0.176$ would be biopsied, whereas patients below this threshold would not.

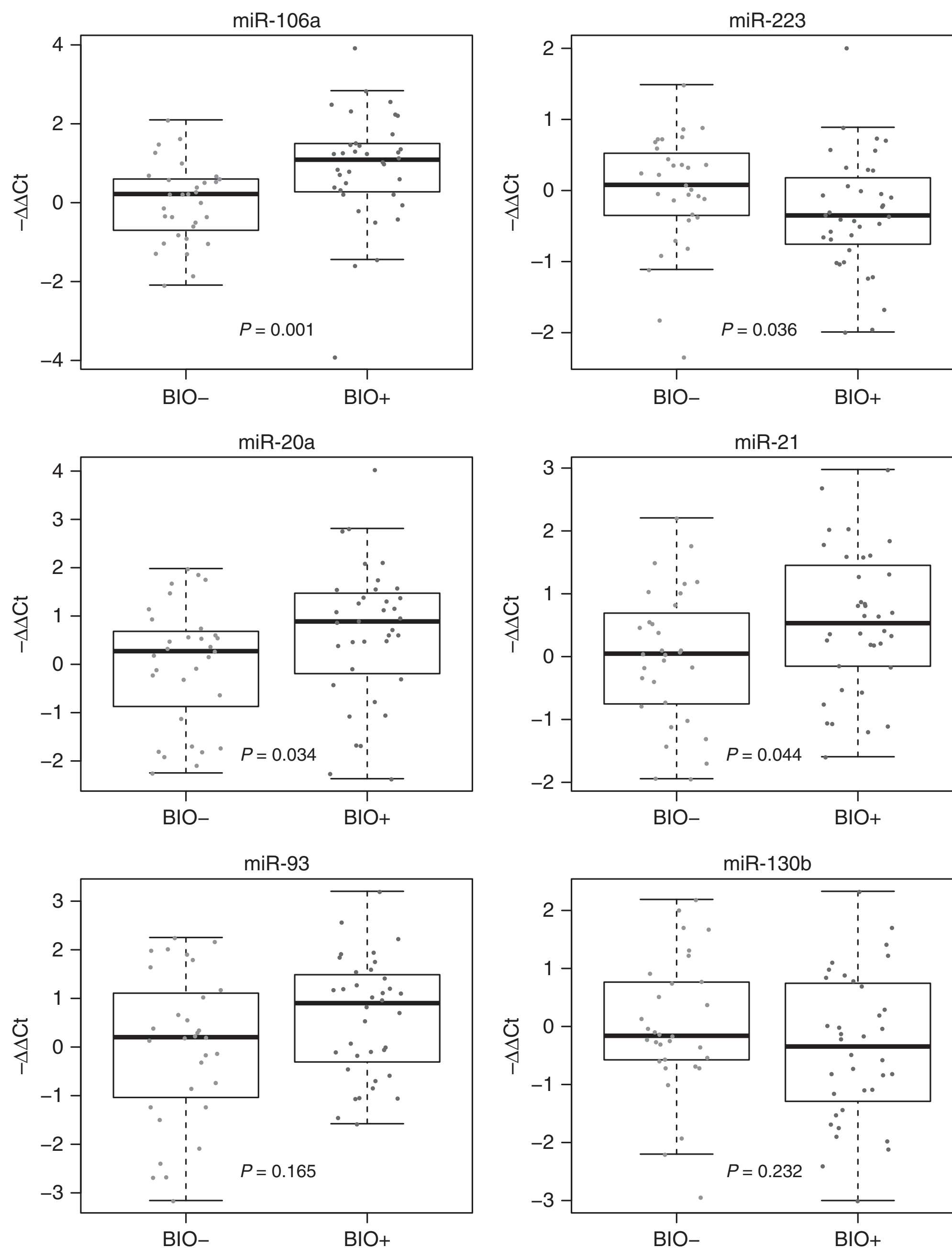

Figure 1. Boxplots of the distribution of –ΔΔCt values for circulating miR-20a, miR-21, miR-93, miR-106a, miR-130b and miR-223 in patients with localised PCa *vs* biopsy-negative individuals. Each dot represents a patient sample. –ΔΔCt values (see Materials and methods) were calculated using endogenous miR-24 as a reference. Differences between BIO + and BIO − samples were analysed using two-tailed Wilcoxon rank-sum test. miR-106a, miR-223, miR-20a and miR-21 showed *P*-values < 0.05.

The left-hand portion of the flowchart refers to patients with PSA $<4\,\mathrm{ng\,ml^{-1}}$, but DRE +. In our data set, among the six patients with these characteristics, the miR-106a/miR-130b ratio correctly distinguished all four BIO + from the two BIO − patients, generating no false-positive or false-negative results. Table 1 compares the diagnostic performance of the proposed biomarkers with PSA.

CONCLUSIONS

Recent large-scale studies found no or very small benefit for periodic PSA and DRE screening after up to 13 years of follow-up (Andriole *et al*, 2012; Schroder *et al*, 2012); identification of other biomarkers to aid early diagnosis and biopsy decision is thus in high demand.

In this study, we explored the efficacy of circulating miRNA as biomarkers for PCa screening. Our findings suggest that a single miRNA might be poorly informative, especially considering the known biases linked to data normalisation. We thus propose to calculate ratios between one upregulated miRNA and one down-regulated miRNA in the same patient. Results showed that the miR-106a/miR-130b and miR-106a/miR-223 ratios discriminated between localised PCa and BPH patients with a specificity much superior to PSA. A diagnostic flowchart that employs two miR ratios in combination with standard PSA (Figure 2C) should considerably increase the specificity and also improve the sensitivity compared with PSA testing alone (Table 1).

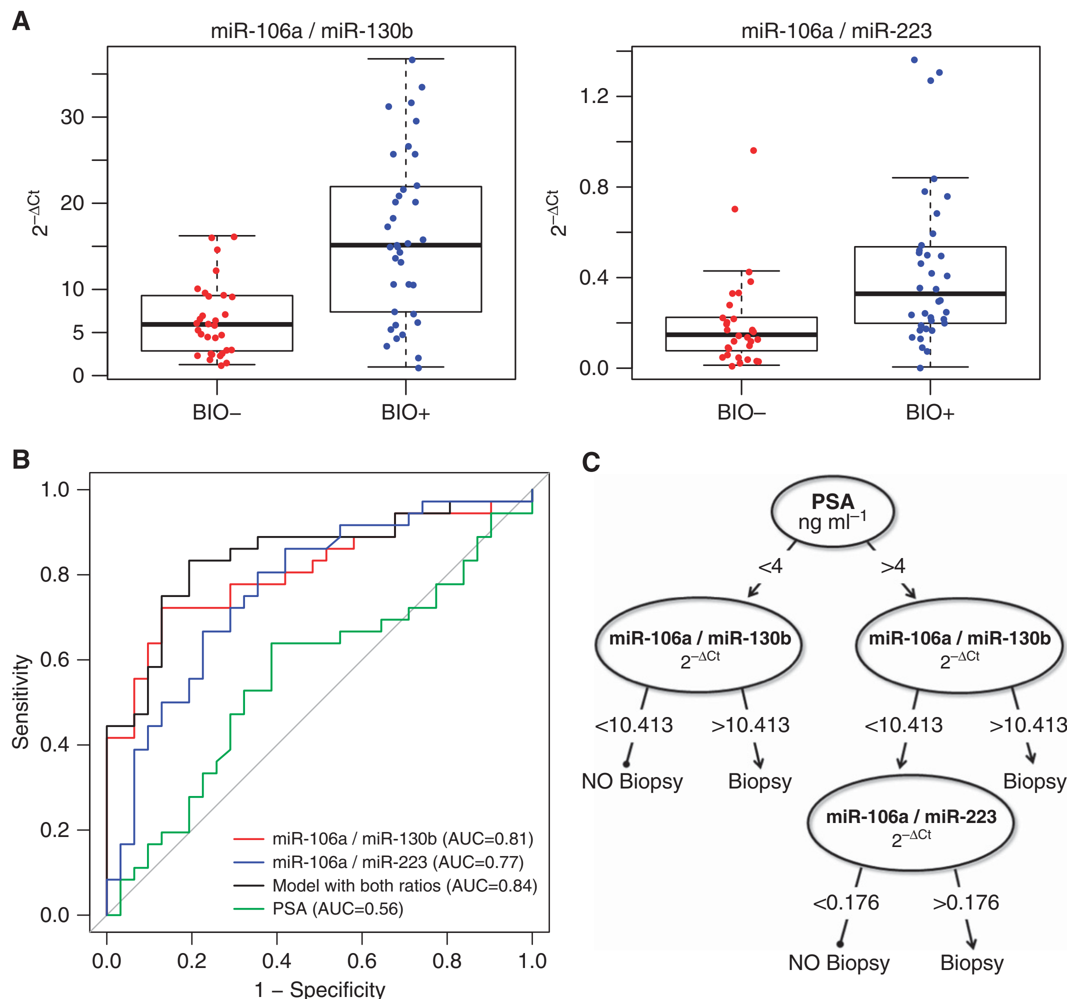

Figure 2. Circulating miR-106a/miR-130b and miR-106a/miR-223 ratios predict the presence of localised prostate cancer. (**A**) Relative expression levels are shown as $2^{-\Delta Ct}$ (see Materials and methods). Both miRNA ratios were significantly higher (one-tailed Wilcoxon rank-sum test) in PCa patients compared with biopsy-negative patients ($P=6.97\times10^{-6}$ for miR-106a/miR-130b; $P=8.5\times10^{-5}$ for miR-106a/miR-223). (**B**) Receiver operating characteristic (ROC) curves generated for univariate (red and blue traces) and bivariate (black trace) logistic regression models against the ROC curve of PSA (green trace). The miRNA ratios were better than PSA for discriminating PCa from BIO – samples. Areas under ROC curves (AUC) are shown in parentheses. (**C**) Proposed miRNA-based diagnostic flowchart for PCa diagnosis.

Table 1. Assessment of the performance of different diagnostic strategies

Biomarker	Threshold	TP	TN	FP	FN	NPV	PPV	Specificity	Sensitivity	Accuracy
miR-106a/miR-130b	10.413	26	27	4	10	0.730	0.867	0.871	0.722	0.791
miR-106a/miR-223	0.176	29	20	11	7	0.741	0.725	0.645	0.806	0.731
miRNA score	2.142	30	25	6	6	0.806	0.833	0.806	0.833	0.821
PSA	4	32	2	29	4	0.333	0.525	0.065	0.889	0.507
Diagnostic flowchart	10.413 and 0.176	33	20	11	3	0.870	0.750	0.645	0.917	0.791

Abbreviations: FN = false negative; FP = false positive; miRNA = microRNA; NPV = negative predictive value; PSA = prostate-specific antigen; PPV = positive predictive value; TP = true positive; TN = true negative. The miR-106a/miR-130b and miR-106a/miR-223 ratios alone or in combination (miRNA score), the diagnostic flowchart (Figure 2C) and standard PSA testing were evaluated for their ability to identify biopsy-positive patients. The table shows the threshold and metrics for the evaluation of each biomarker's performance in our data set.

The miRNA ratio approach described in the present study may be applied to large-scale screening, as it requires measuring only three circulating miRNAs and overcomes the need for a normaliser miRNA. The increased specificity of this assay compared with PSA alone could help to reduce the costs and morbidity of unnecessary repeated biopsies. Future studies should test the validity of this approach in an ample prospective cohort of patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTORS

VC supervised the study. ES and KR performed the RT-PCR analysis. AG designed and performed the statistical analysis. AM, FP, PB, FZ and MI provided blood samples and were responsible for the clinical aspects of the project. PZ provided guidance and support for the study. VC, DMD, ES and AG prepared the manuscript. All the authors contributed to the study design and data interpretation.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)