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 (Andriele 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...
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).

**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 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., 2011). Odds ratios are reported in Supplementary Tables S4 and S5.

**Determined threshold values for miRNA ratios.** Statistical analysis indicated optimal 2−ΔΔCt 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 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., 2011). Odds ratios are reported in Supplementary Tables S4 and S5.

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−ΔΔCt > 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−ΔΔCt > 0.176 would be biopsied, whereas patients below this threshold would not.
The left-hand portion of the flowchart refers to patients with PSA < 4 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 downregulated miRNA in the same patient. Results showed that the miR-106a/miR-130b and 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).
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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