Training and validation of a novel 4-miRNA ratio model (MiCaP) for prediction of postoperative outcome in prostate cancer patients

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Background: New molecular biomarkers for prostate cancer (PC) prognosis are urgently needed. Ratio-based models are attractive, as they require no additional normalization. Here, we train and independently validate a novel 4-miRNA prognostic ratio model for PC.

Patients and methods: By genome-wide miRNA expression profiling of PC tissue samples from 123 men who underwent radical prostatectomy (RP) (PCA123, training cohort), we identified six top candidate prognostic miRNAs and systematically tested their ability to predict postoperative biochemical recurrence (BCR). The best miRNA-based prognostic ratio model (MiCaP) was validated in two independent cohorts (PCA352 and PCA476) including >800 RP patients in total. Clinical end points were BCR and prostate cancer-specific survival (CSS). The prognostic potential of MiCaP was assessed by univariate and multivariate Cox-regression analyses and Kaplan–Meier analyses.

Results: We identified a 4-miRNA ratio model, MiCaP (miR-23a-3p/C2 miR-10b-5p)/(miR-133a/C2 miR-374b-5p), that predicted time to BCR independently of routine clinicopathologic variables in the training cohort (PCA123) and was successfully validated in two independent RP cohorts. In addition, MiCaP was a significant predictor of CSS in univariate analysis [HR 3.35 (95% CI 1.34—8.35), P = 0.0096] and in multivariate analysis [HR 2.43 (95% CI 1.45–4.07), P = 0.0210]. As proof-of-principle, we also analyzed MiCaP in plasma samples from 111 RP patients. A high MiCaP score in plasma was significantly associated with BCR (P = 0.0036, Kaplan–Meier analysis). Limitations include low mortality rates (CSS: 5.4%).

Conclusions: We identified a novel 4-miRNA ratio model (MiCaP) with significant independent prognostic value in three RP cohorts, indicating promising potential to improve PC risk stratification.

Key words: prostate cancer, prognosis, risk stratification, biomarkers, microRNA

Introduction

Prostate cancer (PC) is the most commonly diagnosed cancer in men in the Western world. Currently, serum prostate-specific antigen (PSA) is used for detection of PC and for monitoring of disease progression and treatment response [1, 2]. Over the past decades, extensive use of PSA testing has increased detection rates for early stage tumors, which may be cured by radical prostatectomy (RP) [3]. However, available prognostic indicators (PSA, tumor stage, and Gleason score) are inaccurate and overtreatment is common for these patients, who in reality may have either an aggressive tumor needing immediate intervention, or an indolent tumor that can be managed by active surveillance [4]. There is an urgent need for new molecular biomarkers that can improve risk stratifications for patients with early stage PC, in order to guide more personalized treatment decisions [5].

MicroRNAs (miRNAs) comprise a large class of regulatory noncoding RNAs (~22 nt) that control gene expression by binding to (partially) complementary sequences in target mRNAs, leading to gene silencing [6]. It has been estimated that 60% of all...
human mRNAs are regulated by miRNAs, which thereby influence key cellular processes, e.g. differentiation and proliferation [6, 7]. Furthermore, miRNAs constitute a particularly attractive source for biomarker discovery, as they are more stable than mRNAs and thus easier to extract and quantify from, e.g. formalin-fixed and paraffin-embedded patient tissue samples that have been stored for several years in pathology archives [6, 8]. Although single miRNAs have shown promising potential as diagnostic biomarkers for PC, current evidence suggests that single miRNAs have suboptimal prognostic potential for PC, when compared with multi-miRNA signatures [9, 10]. Ratio-based miRNA models could be particularly useful, as their design precludes the need for additional normalization factors [11].

In this study, we trained and successfully validated a new prognostic 4-miRNA ratio model for prediction of postoperative outcome in PC patients.

Patients and methods

Study design and participants

We used three independent patient cohorts [PCA123 (training set, N = 123), PCA352 (validation set, N = 352), and PCA476 (validation set, N = 476)] of men, who underwent RP for clinically localized PC (Table 1).

PC tissue samples from PCA123 and PCA352 were collected at the Department of Urology, Aarhus University Hospital, Aarhus, Denmark between 1997 and 2005. Clinical follow-up information, including time to BCR, was updated for all patients before this study. Inclusion and exclusion criteria for the cohorts are reported according to the REMARK guidelines (supplementary Figure S1, available at Annals of Oncology online). Written consent was obtained from all participants, and this study was approved by the regional scientific ethical committee and the Danish Data Protection Agency. Total RNA was extracted from archived PC tissue samples and analyzed for miRNA expression using the miRCURY LNA™ Universal RT microRNA PCR platform (Exiqon A/S) (supplementary methods, available at Annals of Oncology online). Assay linearity across a broad concentration range (five orders of magnitude) was confirmed for all 4-miRNA assays by dilution series experiments (supplementary Figure S2, available at Annals of Oncology online). Raw miRNA data for PCA123 can be found online (GEO, with accession number GSE115402).

The PCA476 cohort was collected by the TCGA consortium at multiple centers in the United States and Europe [12, 13]. Normalized miRNA sequencing (small-RNAseq) data and clinical data were retrieved from the TCGA data portal [13] (see supplementary methods, available at Annals of Oncology online).

For miRNA analyses in plasma samples, see supplementary methods, available at Annals of Oncology online.

Ratio model training

A detailed description is found in supplementary methods, available at Annals of Oncology online. In brief, for model training (see flow chart, supplementary Figure S3, available at Annals of Oncology online), we used six top candidate prognostic miRNAs identified by genome-wide miRNA expression profiling of PC tissue samples from 123 RP patients (PCA123; Table 1). Ratio models were stringently trained in PCA123 (supplementary Tables S1–S3, available at Annals of Oncology online), and the prognostic potential of the top candidate 4-miRNA model (MiCaP) (supplementary Table S1, available at Annals of Oncology online) was subsequently tested in two independent validation cohorts: PCA352 and PCA476.

Outcome and statistical analysis

All statistical analyses were conducted in R [14] unless stated otherwise. P values < 0.05 were considered significant. Associations between MiCaP score and clinicopathologic parameters were assessed using Wilcoxon rank-sum and Spearman correlation tests. For evaluation of prognostic potential, the primary clinical end point was BCR-free survival (RFS) after RP. BCR was defined as a postoperative PSA test ≥ 0.2 ng/ml. Patients not having experienced BCR were censored at their last PSA test. For survival analyses, we carried out uni- and multivariable Cox-regression as well as Kaplan–Meier analyses using the ‘survival’ package in R [11]. When relevant, P values were corrected for multiple testing using the Benjamini–Hochberg (BH) method [15]. Predictive accuracy was determined using Harrell’s concordance index (C-index). Decision curve analyses and calibration plots are described in supplementary methods, available at Annals of Oncology online.

Results

MiCaP independently predicts postoperative BCR in three RP cohorts

A systematic procedure was used to train a new 4-miRNA prognostic ratio model MiCaP (miR-23a-3p × miR-10b-5p/miR-133a × miR-374b-5p). The model was strictly trained in the PCA123 cohort and subsequently tested in two independent validation cohorts, including 352 and 476 RP patients, respectively (Table 1; supplementary Tables S1–S3 and Figure S3, available at Annals of Oncology online). A high MiCaP score was significantly associated with advanced pathologic tumor stage, positive surgical margin status, high Gleason score and/or high preoperative PSA in at least one of these cohorts (supplementary Table S4, available at Annals of Oncology online). Furthermore, a significantly higher MiCaP score was observed in PC tissue compared with adjacent nonmalignant prostate tissue in PCA476 (supplementary Table S4, available at Annals of Oncology online).

To assess the prognostic potential of MiCaP, we stratified patients in the training cohort (PCA123) into a high- and a low-risk group based on MiCaP scores. A high MiCaP score was significantly associated with early BCR in univariate Cox-regression analysis, and remained significant in multivariable Cox-regression analysis after adjusting for the CAPRA-S clinical nomogram that includes clinicopathologic variables only (Table 2) [16]. Univariate analysis results for individual clinical variables can be found in supplementary Table S5, available at Annals of Oncology online. In the training cohort PCA123, MiCaP increased the predictive accuracy, as estimated by Harrell’s C-index, from 0.718 to 0.750, compared with the CAPRA-S nomogram only (Table 2).

For independent testing, patients in each of the validation cohorts PCA352 and PCA476 were divided into high- and low-risk groups based on the cut-off (fraction) defined in PCA123. A high MiCaP score was significantly associated with short RFS in univariate Cox-regression analyses in both validation cohorts and remained significant after adjustment for routine clinical parameters using the CAPRA-S nomogram (Table 2). In addition, MiCaP increased the predictive accuracy (C-index) from 0.699 to 0.713 in PCA352, and from 0.661 to 0.687 in PCA476, suggesting improved prognostic power compared with a model based on the CAPRA-S clinical nomogram only (Table 2). Consistent with this, Kaplan–Meier curve analyses showed a...
significant association between a high MiCaP score and short RFS in all three cohorts (Figure 1A–C). To further assess the prognostic potential of MiCaP, we carried out decision curve analysis and calibration plots (supplementary methods, available at Annals of Oncology online). The multivariate model including MiCaP (Table 2) added a modest net benefit for decision-making based on model predictions in all three cohorts (supplementary Figure S4, available at Annals of Oncology online). Furthermore, the multivariate model including MiCaP, divided into three risk-groups, showed strong agreement between observed (Kaplan–Meier estimates) and predicted (Cox-regression model based) outcomes using calibration plots (supplementary Figure S5, available at Annals of Oncology online).

As proof-of-principle, to test the prognostic potential of MiCaP in liquid biopsies, we analyzed plasma samples collected before RP from 111 PC patients. Patients with a high MiCaP score showed significantly shorter time to BCR in Kaplan–Meier analysis (supplementary Figure S6, available at Annals of Oncology online), thereby confirming and expanding on our findings from tissue-based analyses.

**MiCaP predicts cancer-specific survival after RP**

Prostate cancer-specific survival (CSS) analyses could only be carried out for PCA352 due to low event numbers/insufficient follow-up time in PCA123 and PCA476 (Table 1). Patients with a high MiCaP score in PC tissue had significantly shorter CSS in Kaplan–Meier analysis (Figure 1D) and in univariate Cox-regression analysis (HR 3.35, 95% CI 1.34–8.35, \( P = 0.0096 \), Table 3). MiCaP also remained a significant predictor of CSS after adjusting for the CAPRA-S nomogram (HR 2.43, 95% CI 1.45–4.07, \( P = 0.0210 \); Table 3).

**Discussion**

In this study, we systematically trained, tested and validated a novel 4-miRNA prognostic ratio model for PC, named MiCaP.
A high MiCaP score in PC tissue was a significant adverse predictor of BCR beyond routine clinicopathologic variables (as assessed by the CAPRA-S nomogram) in three RP cohorts, comprising more than 950 patients in total. Moreover, a high MiCaP score was significantly associated with shorter prostate CSS independently of the CAPRA-S nomogram [16]. These results suggest that MiCaP might be used in the future to improve risk stratification for patients with clinically localized PC and enable more personalized treatment decisions. To the best of our knowledge, this is the first report of a miRNA-based ratio model for PC with significant independent prognostic value in three distinct PC patient cohorts. Finally, we present proof-of-principle support for MiCaP as a promising minimally invasive biomarker in plasma.

For MiCaP, we found that relatively high expression of miR-10b-5p and miR-23a-3p and relatively low expression of miR-133a and miR-374b-5p in PC tissue samples was associated with poor outcome after RP. Consistent with this, high miR-10b expression has previously been associated with BCR in a small cohort of 52 PC patients [17]. There are no previous reports of a prognostic potential for miR-23a-3p and miR-133a as single markers in PC, while earlier small-scale studies on miR-374b showed contradictory results [18–20], highlighting the importance of using multiple independent and sufficiently sized PC patient cohorts for prognostic biomarker evaluation.

Of the four miRNAs in MiCaP, only miR-133a has previously been included in a multi-miRNA prognostic signature for PC (25-miRNA classifier associated with adverse clinicopathology), but the study lacked independent validation [21]. Other previously proposed models include a 16-miRNA signature [22], a 2-miRNA model [20], and a 3-miRNA classifier [9] for prediction of BCR after RP. However, apart from a single exception [9], all earlier studies lacked independent validation, multivariate analysis, and/or sufficient patient sample size. Furthermore, these proposed multi-miRNA models depend on additional normalization, which is circumvented using a ratio model such as MiCaP and which could potentially ease future translation into clinical practice. Before this study, miQ [(miR-96-5p x miR-183-5p)/(miR-145-5p x miR-221-5p)] was the only tissue-based prognostic miRNA ratio model proposed for PC, but it was not tested in multivariate analysis [10]. In contrast, we found that MiCaP predicted BCR in three distinct RP cohorts independent of routine clinical variables.

The currently used routine prognostic variables (i.e. clinicopathologic parameters and nomograms based on these) for early stage PC cannot accurately predict whether a tumor will progress or remain indolent [5]. Future clinical implementation of improved prognostic biomarkers, such as MiCaP, could help to solve this major challenge in primary PC management by enabling more accurate risk stratification at diagnosis, and thereby better treatment decisions, e.g. between active surveillance or surgery. Risk stratification based on MiCaP could potentially also be useful post-surgery, to assess the need for adjuvant treatment such as radiation or androgen deprivation therapy.

The function of miR-23a-3p, miR-10b-5p, and miR-133a in prostate cancer cells is not fully understood and miR-374b-5p remains to be investigated. MiR-23a and miR-10b promote DU145 PC cell migration [17, 23], while miR-10b was also shown to inhibit proliferation and invasion in PC cells [24]. Two studies report an inhibition of both proliferation and migration in PC3 and DU145 after miR-133a overexpression [25, 26]. Furthermore, overexpression of miR-374b has been shown to inhibit cell proliferation in T-cell lymphoblastic lymphoma [27], whereas a pro-invasive role has been seen in gastric cancer cells [28], together indicating that the function of miR-10b and

| Variable | Characteristic | HR (95% CI) | P-value | C-index | Univariate | HR (95% CI) | P-value | C-index | Multivariate | C-indexa | C-indexb |
|----------|---------------|-------------|---------|---------|------------|-------------|---------|---------|-------------|---------|---------|
| CAPRA-S  | Low           | Ref         | –       | 0.72    | 0.94       | 8.23E-04    | 4.27    | 0.750   | 0.713       | 0.699   |          |
|          | Intermediate  | –           | –       | 0.70    | –          | 3.27       | 5.08E-05| –       | –           | –       |          |
| MiCaP    | Low vs. high  | 3.23        | 0.04    | 0.63    | 1.44       | 2.90E-02   | 2.04    | –       | –           | –       |          |
| PCA352, N = 352, 153 with recurrence | Low          | 3.30 (1.86–5.86) | 8.42E–05 | 3.27 (1.85–5.81) | 5.08E–05 |
|          | High          | 9.43 (5.26–16.90) | 4.97E–14 | 9.25 (5.16–16.59) | 8.30E–14 |
| MiCaP    | Low vs. high  | 1.54 (1.12–2.13) | 8.20E–03 | 1.44 (1.04–2.00) | 2.90E–02 |
| PCA476, N = 405, 58 with recurrence | Low          | 2.04 (1.19–13.72) | 2.53E–02 | 3.28 (0.95–11.37) | 6.06E–02 |
|          | Intermediate  | 9.00 (2.76–29.41) | 2.74E–04 | 6.59 (1.94–22.39) | 2.51E–03 |
| MiCaP    | Low vs. high  | 2.45 (1.46–4.12) | 7.32E–04 | 1.89 (1.08–3.32) | 2.69E–02 |

Significant P values (P < 0.05) are highlighted in bold.

aHarrell’s C-index for final model including ratio model.
bHarrell’s C-index for final model excluding the ratio model.
Figure 1. MiCaP score is associated with BCR and CSS. (A–C) Kaplan–Meier survival analysis of recurrence-free survival (RFS) based on MiCaP scores in three independent RP cohorts. Patients in the training cohort (PCA123) were divided in low- and high-risk groups based on their MiCaP scores. Patients in the validation cohorts (PCA352 and PCA476) were divided into high- and low-risk groups based on the cut-off (fraction) defined in PCA123. A high MiCaP score was significantly associated with shorter RFS in all three cohorts. P values for two-sided log-rank test are given. (D) Kaplan–Meier survival analysis prostate CSS based on MiCaP scores in the PCA352 cohort (n = 352, CSS events = 19). Patients were divided in high- and low-risk groups based on their MiCaP scores. A high MiCaP score was significantly associated with shorter CSS. P value for two-sided log-rank test is given.

Table 3. Uni- and multivariate Cox-regression analysis of CSS using MiCaP

| Variable | Characteristic | Univariate | | | Multivariate | |
|----------|----------------|------------|------------|----------------|------------|-------------|----------------|-------------|-------------|-------------|
|          |                | HR (95% CI) | P-value | C-index | HR (95% CI) | P-value | C-index | C-index | C-index |
| PCA352, N = 352, 19 dead | CAPRA-S Low | Ref | – | 0.73 | 2.74 (0.33–22.84) | 3.51E–01 | 0.783 | 0.734 |
|          | Intermediate | 2.85 (0.34–23.73) | 3.38E–01 | – | 8.90 (1.14–69.69) | 3.74E–02 | 0.783 | 0.734 |
|          | High | 10.15 (1.30–79.37) | 2.72E–02 | – | 8.90 (1.14–69.69) | 3.74E–02 | 0.783 | 0.734 |
|          | Low vs. high | 3.35 (1.34–8.35) | 9.60E–03 | 0.631 | 2.43 (1.45–4.07) | 2.10E–02 | 0.783 | 0.734 |

Significant P values (P < 0.05) are highlighted in bold.

*aHarrell’s C-index for final model including ratio model.

*bHarrell’s C-index for final model excluding the ratio model.
miR-374b-5p is tissue-type dependent. Further studies are needed to unravel the function of miR-374b in nonmalignant and PC cells, as well as of the three other miRNAs included in MiCaP.

Limitations to the present study include different characteristics for the three RP patient cohorts. Clinical follow-up time was shorter in PCA476 (median 15 months) compared with PCA123 and PCA352 (137 and 100 months, respectively). Moreover, different miRNA expression profiling methods were used. PCA123 and PCA352 were profiled using RT-qPCR and PCA476 by small-RNA sequencing. Nevertheless, MiCaP showed significant independent prognostic potential in multivariate analysis in all three cohorts, suggesting it is robust. Furthermore, all PC tissue analyses were based on surgical specimens. Future studies should examine the prognostic potential of MiCaP in prostate needle biopsies to assess if MiCaP can improve risk stratification at the time of diagnosis. Moreover, our study did not address the multifocality/heterogeneity, as we analyzed miRNA expression only in one PC tissue sample (area with highest Gleason grade) from each patient. Thus, future PC tissue-based validation studies of MiCaP should account for possible multifocality/heterogeneity. However, our proof-of-principle study showed promising prognostic potential for MiCaP in plasma samples, although independent validation is needed. In addition, only one cohort was eligible for CSS analysis, in which a low CSS mortality rate was observed (5.4%). Further validation is warranted and should include large patient cohorts with PC-specific survival and >10 years follow-up, as early-stage PC is generally slow-growing [27].

In summary, the new 4-miRNA prognostic model MiCaP independently predicted BCR in three distinct RP cohorts, and was a significant predictor of prostate CSS in an RP cohort comprising 352 patients. Further studies are warranted to assess the true clinical utility of MiCaP for improving risk stratification of clinically localized PC.

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Disclosure

HK, PM, JF, TO, and KDS are co-inventors on patent application(s) regarding miRNAs as biomarkers for prostate cancer. KDS has received consultancy fees from Exiqon A/S. All remaining authors have declared no conflicts of interest.

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