Downregulation and Prognostic Performance of MicroRNA 224 Expression in Prostate Cancer

Konstantinos Mavridis, Konstantinos Stravodimos, and Andreas Scorilas

INTRODUCTION: The extensive use of prostate-specific antigen as a general prostate cancer biomarker has introduced the hazards of overdiagnosis and overtreatment. Recent studies have revealed the immense biomarker capacity of microRNAs (miRNAs) in prostate cancer. The aim of this study was to analyze the expression pattern of miR-224, a cancer-related miRNA, in prostate tumors and investigate its clinical utility.

METHODS: Total RNA was isolated from 139 prostate tissue samples. After the polyadenylation of total RNA by poly(A) polymerase, cDNA was synthesized with a suitable poly(T) adapter. miR-224 expression was assessed by quantitative real-time PCR and analyzed with the comparative quantification cycle method, $C_q(2^{\Delta C_q})$. We performed comprehensive biostatistical analyses to explore the clinical value of miR-224 in prostate cancer.

RESULTS: miR-224 expression was significantly down-regulated in malignant samples compared with benign samples ($P \leq 0.001$). Higher miR-224 expression levels were found in prostate tumors that were less aggressive ($P = 0.017$) and in an earlier disease stage ($P = 0.018$). Patients with prostate cancer who were positive for miR-224 had significantly enhanced progression-free survival intervals compared with miR-224–negative patients ($P = 0.021$). Univariate bootstrap Cox regression confirmed that miR-224 was associated with favorable prognosis (hazard ratio 0.314, $P = 0.013$); nonetheless, multivariate analysis, adjusted for conventional markers, did not identify miR-224 as an independent prognostic indicator.

CONCLUSIONS: miR-224 is aberrantly expressed in prostate cancer. Its assessment by cost-effective quantitative molecular methodologies could provide a useful biomarker for prostate cancer.

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and CaP with a reliable and cost-efficient method based on quantitative real-time PCR (qPCR). We chose tissue samples from patients with BPH as a control group to provide an initial assessment of miR-224 as a potential discriminator of malignant from common benign tumors. We also investigated the prognostic performance of miR-224 in CaP with respect to prediction of disease progression. We selected miR-224 for analysis because of its repeatedly reported aberrant expression in various human malignancies (8–22) and its capacity for targeting numerous cancer-related genes.

Materials and Methods

PROSTATE TISSUE SAMPLES
This study was carried out according to the ethical principles of the 1975 Declaration of Helsinki, as revised in 2008. Approval for the use of the prostate tissue samples was acquired by the ethical committee of “Laiko” University Hospital. Informed consent was provided by all participating patients.

We analyzed 139 snap-frozen tumor tissue samples from patients with BPH (n = 66) or CaP (n = 73) (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue1), collected at the “Laiko” University Hospital, Athens, Greece. Patients with BPH had undergone surgery with either a transurethral or open prostatectomy method, whereas radical prostatectomy was performed in patients with CaP. No hormonal treatment or radiotherapy was given to the patients of this study before surgery. A tissue sample of approximately 200 mg was sectioned from the peripheral zone of the prostate gland of patients with CaP on the basis of the preoperative features of the biopsy and macroscopic findings. Subsequently, with the purpose of corroborating the presence of malignancy, two mirror-image segments were produced and one part of each sample was assessed by the same pathologist. The remaining part was snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Only the tissue parts that were confirmed as malignant from CaP, as well as the tissue samples obtained from the adenomectomy of patients with BPH, were used for subsequent analysis. Fifteen patients were excluded from the survival analysis because of (a) unavailability at follow-up; (b) unavailability of precise monitoring information; or (c) administration of adjuvant therapy, after radical prostatectomy and before any biochemical relapse, to high-risk patients (e.g., individuals with positive surgical margins).

PSA MEASUREMENTS
For preoperative and postoperative PSA measurements, 5 mL venous blood was sampled and left to clot at room temperature for 1 h. After centrifugation of the blood samples at 2000g for 15 min, serum samples were collected and stored at −70 °C until measurement. We measured PSA serum concentrations with the commercially available PSA-RIACT™ immunoradiometric assay kit (Cis Bio International), according to the manufacturer’s instructions. Intraassay and interassay CVs of the above method were 2.4% and 3.9%, respectively. Serum PSA concentrations after radical prostatectomy for all patients included in the survival analysis were <0.1 µg/L. The time to biochemical relapse was defined as the period between surgery and the persistent increase of serum PSA concentrations, evidenced by 2 consecutive PSA results ≥0.2 µg/L.

RNA EXTRACTION
We extracted total RNA from prostate tissue samples, after pulverization and homogenization, as well as from the LNCaP cells, with the TRI reagent® (Molecular Research Center), according to the manufacturer’s instructions. The resulting RNA pellet was dissolved in RNA Storage Solution (Applied Biosystems/Ambion) and stored in aliquots at −80 °C until use. We determined total RNA concentration and purity spectrophotometrically and confirmed RNA integrity by agarose gel electrophoresis.

POLYADENYLATION AND cDNA SYNTHESIS
Isolated total RNA (1 µg), including miRNAs, was polyadenylated in the presence of ATP (80 µmol/L) by 1 U poly(A) polymerase (New England Biolabs) at 37 °C for 1 h. The reaction buffer consisted of 50 mmol/L Tris-HCl, 250 mmol/L NaCl, and 10 mmol/L MgCl₂. The enzyme was heat-inactivated at 65 °C for 10 min.

The resulting polyadenylated RNA was then mixed with poly(T) adapter, 5′-GGGAGACACAGATTAATACGACTCTATAGTGTGTTTTTTTTTTTN-3′ (0.25 µmol/L) where V = G, A, C and N = G, A, T, C, heated at 70 °C for 5 min, and quick-cooled on ice to denature any secondary structures and allow the annealing of the poly(T) adapter. We performed reverse transcription into cDNA by adding 100 U Moloney murine leukemia virus reverse transcriptase (Invitrogen), 40 U recombinant ribonuclease inhibitor (Invitrogen), and the reaction buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl₂, 10 mmol/L dithiothreitol, and 0.5 mmol/L of each dNTP). The final reaction volume was 20 µL. We terminated reverse transcription (37 °C for 60 min) by incubation at 70 °C for 15 min.

qPCR
On the basis of published sequences of mature miR-224 and SNORD48 (small nucleolar RNA, C/D box 48)
Clinical Performance of miR-224 in Prostate Cancer

Quantitative Assessment of miR-224 Expression Levels in Prostate Tumors

Confirmation of specific amplification of the preferred amplexons is presented in online Supplemental Fig. 1, evidenced by a unique peak at the melting curve analysis (miR-224 product $T_m = 73.3 \degree C$, SNORD48 product $T_m = 79.6 \degree C$) and the detection of a single distinctive band by agarose gel electrophoresis for randomly selected prostate tissue samples. The efficiencies of miR-224 and SNORD48 amplification, calculated from the slopes ($-3.562, r^2 = 0.9996$, and $-3.482, r^2 = 0.9955$, respectively) of the curves deriving from the validation experiments, were 91% and 94%. This proves that the PCR amplexons were produced with similar efficiencies and consequently allowed the use of the $\Delta \Delta C_q$ calculation method. The intraassay CV of the
expression units (RQ) was 2.92%, whereas the interassay CV ranged from 6.29% to 9.13%.

DOWNREGULATION OF miR-224 EXPRESSION IN BPH COMPARED TO CaP SAMPLES

The expression of miR-224 was significantly decreased ($P < 0.001$) in CaP compared with BPH samples (Fig. 1A; Table 1). The median miR-224 expression level was 2.145 RQ units in the malignant and 3.706 RQ units in the benign prostate tissue samples (Fig. 1A; Table 1).

We used ROC curve analysis to evaluate the discriminatory potential of this miRNA for patients with BPH and CaP. miR-224 could distinguish patients with CaP from patients with BPH with an AUC of 0.672 (95% CI 0.583–0.761, $P < 0.001$) (Fig. 1B). The corresponding AUC for PSA was 0.785 (95% CI 0.695–0.875, $P < 0.001$).

We further corroborated the discriminatory value of miR-224 by both univariate and multivariate logistic regression analyses (Table 2). Men who presented with increased levels of miR-224 expression became less likely (crude odds ratio 0.534, 95% CI 0.322–0.884) to have CaP ($P = 0.015$). Corresponding multivariate models, also corrected for serum PSA concentrations and digital rectal exam status, which are the most important indicators of prostate malignancy, revealed the independent discriminatory capacity of miR-224 expression (crude odds ratio 0.381, 95% CI 0.168–0.864, $P = 0.021$) (Table 2).

![Fig. 1.](A), Downregulation of miR-224 in patients with CaP compared to patients with BPH. Bold lines represent the median value. *Outliers. (B), ROC curve analysis for miR-224 expression levels.

**Table 1. Distribution of miR-224 expression levels, serum PSA concentrations, and age in patients with CaP or BPH.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SE</th>
<th>Range</th>
<th>Percentile</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>10th</td>
<td>25th (median)</td>
</tr>
<tr>
<td>Patients with CaP (n = 73)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-224 expression, RQ*</td>
<td>4.228 ± 0.809</td>
<td>0.086–46.64</td>
<td>0.3632</td>
</tr>
<tr>
<td>PSA, ng/mL</td>
<td>9.23 ± 0.69</td>
<td>2.20–41.8</td>
<td>4.17</td>
</tr>
<tr>
<td>Age, years</td>
<td>64.8 ± 0.74</td>
<td>52–76</td>
<td>56</td>
</tr>
<tr>
<td>Patients with BPH (n = 66)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-224 expression, RQ*</td>
<td>8.470 ± 2.09</td>
<td>0.4090–118.7</td>
<td>1.127</td>
</tr>
<tr>
<td>PSA, ng/mL</td>
<td>5.11 ± 0.72</td>
<td>0.400–25.6</td>
<td>1.10</td>
</tr>
<tr>
<td>Age, years</td>
<td>69.6 ± 0.95</td>
<td>49–86</td>
<td>60</td>
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*Normalized to SNORD48 expression.
miR-224 EXPRESSION IN RELATION TO CLINICOPATHOLOGICAL VARIABLES

miR-224 expression gradually decreased in patients with more aggressive tumors \( (P = 0.017) \) (Fig. 2A). Median miR-224 expression in the group of patients with Gleason score \( \leq 6 \) was 2.63 RQ units, and it decreased progressively in Gleason score 7 (median 1.84 RQ units) and Gleason score \( > 7 \) (median 0.725 RQ units) prostate tumors. Additionally, patients with advanced disease, indicated by pathological stage \( \geq \text{pT}2\text{c} \), where the tumor has progressed to both prostatic lobes or even further, showed significantly \( (P = 0.018) \) lower miR-224 expression (median 1.27 RQ units) compared with pathological stage \( < \text{pT}2\text{c} \) patients (median 2.63 RQ units), where the malignancy is confined in one prostatic lobe (Fig. 2B). The negative correlation between miR-224 expression and both greater Gleason score and advanced disease stage was also confirmed by Spearman analysis \( (r_s = -0.307, P = 0.009, \text{ and } r_s = -0.299, P = 0.010, \text{ respectively}) \). Moreover, miR-224 expression levels correlated negatively with serum PSA concentrations \( (r_s = -0.284, P = 0.015) \) in patients with CaP; a negative correlation was also observed in patients with BPH, but lacked statistical significance \( (r_s = -0.275, P = 0.056) \). No correlation was observed between miR-224 and age.

miR-224 AS A BIOMARKER OF FAVORABLE PROGNOSIS FOR CaP PATIENTS

We used Kaplan–Meier progression-free survival curve analysis (Fig. 3) as a first step of investigating the prognostic properties of miR-224 in prostate cancer. Disease progression was identified by biochemical relapse. Patients with CaP categorized as miR-224–positive (according to the median expression) had a superior progression-free survival course compared with miR-224–negative patients \( (P = 0.021) \). The 5-year cumulative probability of progression-free survival for

![Fig. 2. Expression of miR-224 in prostate tumors with Gleason Score \( \leq 6 \) (n = 24), 7 (n = 39), and \( > 7 \) (n = 9) (A) and early (n = 34) vs advanced (n = 39) pathological stage tumors (B). *Outliers.](image-url)
miR-224–positive patients was 0.797 (0.081) compared with only 0.438 (0.094) for miR-224–negative patients (Fig. 3).

Bootstrap Cox proportional hazards regression analysis, at the univariate level, confirmed the favorable prognostic properties of miR-224. As the expression levels of miR-224 increased, patients with CaP showed a statistically significant ($P = 0.013$) decreased risk of relapse over time [hazard ratio (HR) 0.314, 95% CI 0.118–0.712] (Table 3). The above model was also adjusted for miR-224 expression, Gleason score, pathological stage, and preoperative PSA values. In this multivariate model, only Gleason score retained a strong independent prognostic value (HR 2.054, 95% CI 0.975–4.91, $P = 0.022$) (Table 3).

**Discussion**

miRNAs are well known for their implication in cancer (6). These molecules could also introduce a new era of diagnostic, prognostic, and therapeutic modalities for CaP (3–5), the management of which is affected by the complexities of PSA testing (1, 2). To our knowledge, this is the first study to comprehensively analyze the expression of miR-224 in prostate tumors and provide an initial investigation of its biomarker potential for CaP.

miR-224 is aberrantly expressed in several human neoplasms (8–22) and is characterized by contradictory properties, since it can promote (28, 29) or inhibit (30, 31) cancer cell growth, depending on the malignancy type. miR-224 is upregulated in thyroid tumors.

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**Table 3.** Cox proportional hazards regression analysis of miR-224 expression and clinicopathological variables for the prediction of progression-free survival, based on 2000 bootstrap samples.

<table>
<thead>
<tr>
<th>Covariant</th>
<th>Univariate analysis</th>
<th>Multivariate analysis*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HR 95% Bootstrap</td>
<td>Bootstrap P value</td>
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<tr>
<td></td>
<td>BCa CI a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HR 95% Bootstrap</td>
<td>Bootstrap P value</td>
</tr>
<tr>
<td></td>
<td>BCa CI a</td>
<td></td>
</tr>
<tr>
<td>Log$_{10}$(miR-224)</td>
<td>0.314</td>
<td>0.013</td>
</tr>
<tr>
<td></td>
<td>0.118–0.712</td>
<td></td>
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<tr>
<td>Gleason score (ordinal)</td>
<td>2.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>1.51–6.02</td>
<td></td>
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<tr>
<td>Pathological stage (ordinal)</td>
<td>1.47</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>1.10–2.13</td>
<td></td>
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<tr>
<td>Preoperative PSA</td>
<td>1.13</td>
<td>0.001</td>
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<tr>
<td></td>
<td>1.02–1.32</td>
<td></td>
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</tbody>
</table>

* Adjusted for log$_{10}$miR-224 expression, Gleason score, pathological stage and preoperative PSA.

BCa, bias corrected and accelerated.
aggressive pancreatic ductal adenocarcinoma (14); clear cell renal cell carcinoma (15); bladder (20), hepatocellular (21) and colon cancers (11); a subgroup of leukemia patients (18); and perineural invasion-related prostate tumors (19). Conversely, miR-224 is downregulated, compared to nonmalignant controls, in ovarian (9), lung (8), prostate (22), and breast cancers (12); malignant giant cell tumor cells (17); and oral carcinoma (13).

The multifaceted involvement of miR-224 in malignancy can be explained by the fact that it targets the expression of several cancer-associated genes. The validated target genes for miR-224 are API5 (apoptosis inhibitor 5) (21), AP2M1 (adaptor-related protein complex 2, mu subunit) (33), CDC42 [cell division cycle 42 (GTP binding protein, 25 kDa)], CXCR4 [chemokine (C-X-C motif) receptor 4] (30), SMAD4 (SMAD family member 4) (34), SMAD5 (SMAD family member 5) (17), SLMAP (sarcolemma-associated protein) (17), H3.3B [H3 histone, family 3B (H3.3B)] (17), DIO1 (deiodinase, iodothyronine, type 1) (16), DMN1 (33), VHL (von Hippel–Lindau tumor suppressor, E3 ubiquitin protein ligase) (35), HIF1A [hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)] (35), KLLK10 (kallikrein-related peptidase 10) (31), and KLK1 (kallikrein 1) (36). As far as CaP is concerned, CXCR4 is overexpressed in prostate malignancies, probably through posttranscriptional regulation (37), and is associated with the metastatic progression of the disease (38). HIF1A is also highly expressed in cancerous prostate cells and is associated with their malignant phenotypes (39). KLK15 (kallikrein-related peptidase 15), a cancer-related serine protease gene belonging to the same gene family as PSA (40), is identified among the most probable predicted targets of miR-224 (31, 36). Our group and other research groups have demonstrated the marked upregulation of KLK15 in CaP and its association with progressed disease (40).

In the present study, we revealed that miR-224 is significantly downregulated in CaP compared with BPH. Taking a step further, we also showed that the analysis of miR-224 expression levels can discriminate patients with CaP from patients with BPH, as indicated by ROC analysis, as well as both univariate (P = 0.015) and multivariate (P = 0.021) binary logistic regression models. Recent studies conducted on malignant and normal adjacent prostate tissue samples have shown that several other miRNA molecules (e.g., miR-222, -205, -183, -145), and especially their combinatory use, can provide important diagnostic information with an enhanced AUC (7, 22) compared with that calculated in this study. Additionally, the AUC of miR-224 seems inferior when directly compared to established noninvasive prostate cancer biomarkers such as PSA or prostate cancer antigen 3 (1). Additional points for consideration are that our analysis was performed after an invasive procedure and that this analysis cannot substitute for the histological result of the biopsy. It would be interesting to analyze miR-224 expression at a larger scale, in combination with other miRNAs, and to include appropriate serum samples to clearly examine the differential diagnostic capacity of miR-224. Another finding of the present study was that miR-224 expression levels were not correlated with and thus not affected by age in the CaP or the BPH subgroup. Consequently, the fact that patients with BPH had a higher mean age than patients with CaP (Table 1) could not influence the comparisons regarding miR-224 expression.

As far as miR-224 expression in malignant prostate tumors is concerned, this miRNA was found to be associated with both less advanced (P = 0.018) and less aggressive (P = 0.017) disease. A statistically significant negative correlation (r = -0.284, P = 0.015) between miR224 expression and total serum PSA concentrations was also observed. Taken together, these findings provide an initial association between increased miR-224 expression levels and more favorable disease characteristics. High miR-224 expression is also associated with a more favorable outcome for patients with CaP, as shown by Kaplan–Meier progression-free survival curves (P = 0.021) and univariate bootstrap Cox regression analysis (P = 0.013). Nonetheless, when miR-224 expression was used in a multivariate model along with important conventional indicators of CaP progression (namely Gleason score, pathological stage, and PSA), only Gleason score emerged as an independent prognostic marker (P = 0.022). A first look at these results reveals the general prognostic superiority of the Gleason score and the rather limited prediction performance of miR-224 compared to this traditional marker. However, even such consistent indicators as Gleason score may not always provide meaningful information for all patients, given the vast heterogeneity of the disease. The clinical utility of miR-224 in CaP is yet to be extensively explored, since this is the first report that evaluates its capacity as a CaP biomarker. Although our findings do not provide sufficient justification for the direct use of miR-224 in routine clinical decision-making, they are encouraging for further related research efforts. Future directions could include the study of miR-224 in patient subgroups having an unforeseen disease course when conventional prognostic indicators are used.

The above findings may partially contradict with the study of Prueit et al. (19) regarding miR-224 over-
expression in perineural invasion–related compared to non–perineural invasion–related prostate tumors. However, their observation refers to only a subset of invasive prostate tumors and uses different methods from the ones described herein. In agreement with our results, a recent study states that miR-224 is downregulated in cancerous compared with normal adjacent prostate tissue (22). The role of miR-224 as a biomarker of favorable prognosis has also been revealed in medullary thyroid carcinoma, where high miR-224 expression is related with earlier disease stages and superior patient outcome (10).

In conclusion, we demonstrate that miR-224 is downregulated in CaP compared with BPH, that miR-244 expression is gradually decreased as malignancy progresses, and that miR-224 expression is associated with favorable prognosis. Nonetheless, our study did not identify miR-224 as an independent prognostic indicator. Additional studies are thus warranted to evaluate more broadly the potential of miR-224 as a prostate cancer biomarker.

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References

28. Li X, Shen Y, Ichikawa H, Antes T, Goldberg GS. Regulation of microRNA expression by SRC and con-