Clinical Chemistry 59:1
261–269 (2013)

Downregulation and Prognostic Performance of MicroRNA 224 Expression in Prostate Cancer

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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, $C_q(2^{-\Delta\Delta Cq})$. We performed comprehensive biostatistical analyses to explore the clinical value of miR-224 in prostate cancer.

Results: miR-224 expression was significantly downregulated in malignant samples compared with benign samples ($P < 0.001$). 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.

Prostate-specific antigen (PSA)[3] is one of the most extensively used cancer biomarkers *(1)*. Nevertheless, the importance of PSA in prostate cancer (CaP) screening and prognosis is still being challenged *(1, 2)*, since any benefits of PSA testing come with the costs of overdiagnosis and overtreatment. Apart from the undoubted physiological and psychological inconvenience for the patients, the increased financial costs for healthcare systems globally should be taken into account as well, considering also the high prevalence of prostate malignancies *(1, 2)*. Consequently, there is an urgent need for novel CaP biomarkers.

Numerous studies illustrate that microRNAs (miRNAs) are aberrantly expressed in human malignancies, including CaP *(3–5)*. Because miRNAs exert key roles in carcinogenesis and tumor progression, miRNA genes (*MIR*)[4] are currently considered a novel class of oncogenes or tumor suppressor genes *(6)*. Moreover, miRNAs are regarded as promising biomarkers and therapeutic tools for human neoplasms, including urologic tumors *(3)*. Recent studies describe the important diagnostic, prognostic, and predictive role of miRNAs in CaP *(3, 4)*. Nonetheless, the majority of these studies are based on microarray expression data that are characterized by inconsistencies, since no significant overlap has been reported between them *(5, 7)*.

The aim of this study was to comprehensively profile the expression of the mature miRNA 224 (miR-224) molecule in benign prostatic hyperplasia (BPH)

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Received June 15, 2012; accepted October 22, 2012.
Previously published online at DOI: 10.1373/clinchem.2012.191502
[3] Nonstandard abbreviations: PSA, prostate-specific antigen; CaP, prostate cancer; miRNA, microRNA; miR-224, miRNA 224; BPH, benign prostatic hyperplasia; qPCR, quantitative real-time PCR; SNORD48, small nucleolar RNA, C/D box 48; C_q, quantification cycle; RQ, relative quantification; AUC, area under the curve; HR, hazard ratio.
[4] Human genes: *MIR*, microRNA gene family; *API5*, apoptosis inhibitor 5; *AP2M1*, adaptor-related protein complex 2, mu 1 subunit; *CDC42*, cell division cycle 42 (GTP binding protein, 25 kDa); *CXCR4*, chemokine (C-X-C motif) receptor 4; *SMAD4*, SMAD family member 4; *SMAD5*, SMAD family member 5; *SLMAP*, sarcolemma-associated protein; *H3.3B*, H3 histone, family 3B (H3.3B); *DIO1*, deiodinase, iodothyronine, type I; *VHL*, von Hippel–Lindau tumor suppressor, E3 ubiquitin protein ligase; *HIF1A*, hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor); *KLK10*, kallikrein-related peptidase 10; *KLK1*, kallikrein 1; *KLK15*, kallikrein-related peptidase 15.

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 2000*g* 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 $MgCl_2$. The enzyme was heat-inactivated at 65 °C for 10 min.

The resulting polyadenylated RNA was then mixed with poly(T) adapter, 5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-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 $MgCl_2$, 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)

(GenBank accession numbers NR_029638 and NR_002745, respectively), we designed 2 specific forward primers. The sequences of the miR-224–specific primer was 5′-CAAGTCACTAGTGGTTCCGTTAA-3′, and that of the SNORD48-specific primers was 5′-TGATGATGACCCCAGGTAACTCT-3′. We used a sequence hybridizing to the poly(T) adapter (5′-GCGAGCACAGAATTAATACGAC-3′) as the reverse primer. The resulting PCR amplicons during miR-224 and SNORD48 amplification were 65 and 105 bp long, respectively. We selected SNORD48 (RNU48) for normalization purposes owing to its broad use as a reference molecule for miRNA expression studies in CaP and its reported biological stability *(23–26)*. We also found that SNORD48 expression was not significantly different between tissue samples from patients with BPH and patients with CaP or between samples from patients with various disease stages or Gleason scores. Quantitative real-time PCR was carried out with the SYBR® green chemistry, in 96-well PCR microplates (Axygen) placed in a 7500 Real-Time PCR System (Applied Biosystems). We assessed the fluorescence emission of the products and subsequent calculations by use of Sequence Detection System software (Applied Biosystems). The reaction mixture (10 μL total volume per well) included 2 ng cDNA, 2.5 μL diethylpyrocarbonate-treated water (Applied Biosystems), 5 μL Kapa SYBR Fast Universal 2X qPCR Master Mix (Kapa Biosystems), 0.2 μL of 50× Rox Low passive reference dye (Kapa Biosystems), and primers at a final concentration of 200 nmol/L. The reactions were performed in duplicate under the following conditions: 95 °C for 3 min as an initial step followed by 40 cycles of 95 °C for 15 s and 59 °C for 60 s. After amplification, we produced dissociation curves (59 °C to 95 °C at a heating rate of 0.1 °C/sec and acquiring fluorescence data every 0.3 °C) to discriminate the main reaction products from other nonspecific ones or primer-dimers. Furthermore, we subjected randomly selected miR-224 and SNORD48 PCR products to electrophoresis on 3% wt/vol agarose gels to corroborate the presence of a unique amplicon. Each qPCR run always included a no-cDNA template control. Additionally, reverse transcription negative controls were tested and the use of DNA as a qPCR template was analyzed. All the aforementioned negative controls gave no detectable quantification cycle (C_q) value, corroborating the lack of any contamination or nonspecific signal.

We computed the results by the comparative $C_q(2^{-\Delta\Delta Cq})$ method. The normalized results of the expression analysis are presented as relative quantification (RQ) units ($2^{-\Delta\Delta Cq}$) demonstrating the expression ratio of miR-224/SNORD48. ΔC_q is equal to the difference between the C_q of the target molecule (miR-224) and the threshold cycle of the corresponding endogenous reference (SNORD48) in each sample. $\Delta\Delta C_q$ is the difference between the ΔC_q values of a test sample and the calibrator sample (LNCaP cells). We conducted a validation experiment to test the requirements for applying the above methodology. A dilution series of control cDNA, incorporating several orders of magnitude, was amplified, and C_q values were plotted against $\log_{10}$[cDNA quantity]. We estimated reaction efficiency (E) with the formula, $E\% = [-1 + 10^{(-1/\text{slope})}] \times 100$. We also determined the intraassay and interassay CVs of the RQ units for this qPCR assay.

STATISTICAL ANALYSES

We used nonparametric Mann–Whitney U and the Kruskal–Wallis statistical tests to analyze the differences of miR-224 expression values between patient groups. Prediction of the presence of CaP was performed by use of univariate and multivariate binary logistic regression models. We determined associations between the continuous variables of the study by use of Spearman correlation coefficient (r_s). Survival analyses were performed with Kaplan–Meier curves, dichotomized miR-224 status according to the median expression in CaP (2.145 RQ units), and the log-rank statistical test for comparison, as well as bootstrap Cox proportional hazards regression models. An ROC curve was generated under the nonparametric distribution assumption for miR-224 expression levels by plotting sensitivity vs (1 − specificity). The outcome determined was the presence of CaP (test direction: smaller test result indicates more positive test). The calculations for the area under the curve (AUC) were based on the method of Hanley and McNeil *(27)*. We performed statistical analyses with IBM SPSS® software, version 20.0.0; P values <0.05 were considered statistically significant.

Results

QUANTITATIVE ASSESSMENT OF miR-224 EXPRESSION LEVELS IN PROSTATE TUMORS

Confirmation of specific amplification of the preferred amplicons is presented in online Supplemental Fig. 1, evidenced by a unique peak at the melting curve analysis (miR-224 product $T_m = 73.3$ °C, SNORD48 product $T_m = 79.6$ °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 amplicons were produced with similar efficiencies and consequently allowed the use of the $\Delta\Delta C_q$ calculation method. The intraassay CV of the

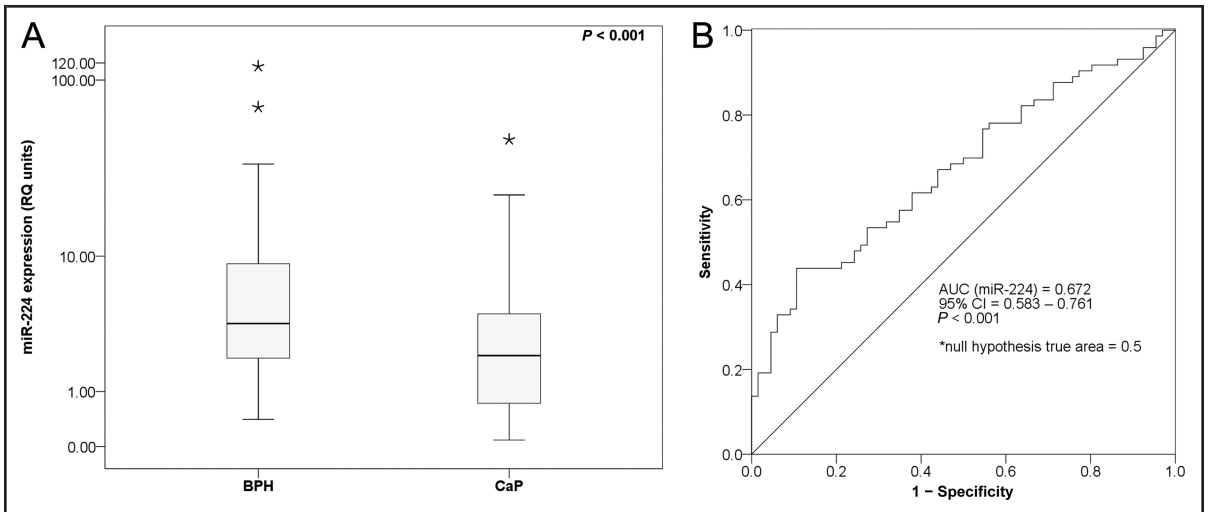

Fig. 1. (A), Downregulation of miR-224 in patients with CaP compared to patients with BPH.
Bold lines represent the median value. *Outliers. (B), ROC curve analysis for miR-224 expression levels.

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).

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

			Percentile				
Variable	Mean ± SE	Range	10th	25th	50th (median)	75th	90th
Patients with CaP (n = 73)							
miR-224 expression, RQ[a]	4.228 ± 0.809	0.086–46.64	0.3632	0.7070	2.145	4.327	10.32
PSA, ng/mL	9.23 ± 0.69	2.20–41.8	4.17	5.54	7.65	11.6	15.6
Age, years	64.8 ± 0.74	52–76	56	60	64	70	74
Patients with BPH (n = 66)							
miR-224 expression, RQ[a]	8.470 ± 2.09	0.4090–118.7	1.127	1.994	3.706	9.139	15.21
PSA, ng/mL	5.11 ± 0.72	0.400–25.6	1.10	1.59	3.89	6.14	12.2
Age, years	69.6 ± 0.95	49–86	60	65	70	75	80

[a] Normalized to SNORD48 expression.

Table 2. Binary logistic regression analysis for the occurrence of CaP.

	Univariate analysis			Multivariate analysis		
Covariant	Crude odds ratio	95% CI	*P*[a]	Crude odds ratio	95% CI	*P*[a]
Log_{10}(miR-224)	0.534	0.322–0.884	0.015	0.381	0.168–0.864	0.021
PSA	1.10	1.04–1.15	<0.001	1.04	0.969–1.12	0.274
Negative digital rectal exam	1.00			1.00		
Positive digital rectal exam	22.0	5.33–90.8	<0.001	21.1	4.63–95.9	<0.001

[a] Test for trend.

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 ≤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 ≥pT2c, 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 <pT2c 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 ($r_s = -0.307$, $P = 0.009$, and $r_s = -0.299$, $P = 0.010$, 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

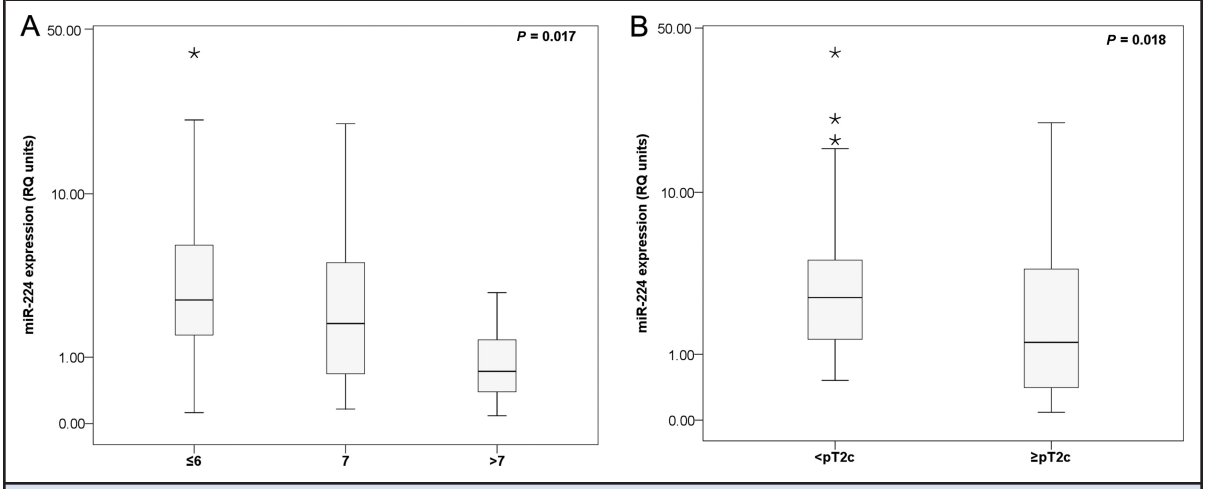

Fig. 2. Expression of miR-224 in prostate tumors with Gleason Score ≤6 (n = 24), 7 (n = 39), and >7 (n = 9) (A) and early (n = 34) vs advanced (n = 39) pathological stage tumors (B).

*Outliers.

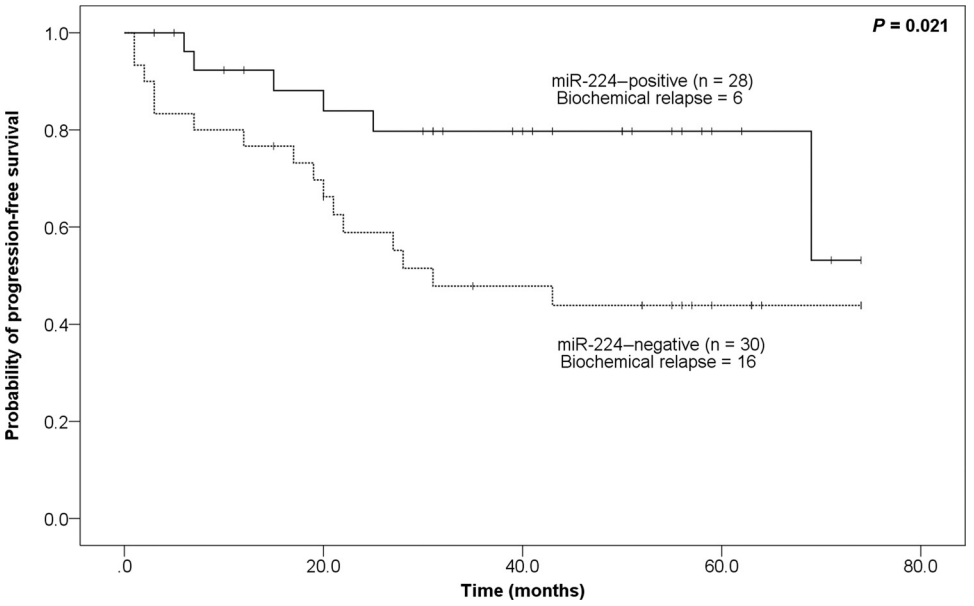

Fig. 3. Kaplan–Meier progression-free survival curves for miR-224–positive and –negative patients.

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

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.

	Univariate analysis			Multivariate analysis[a]		
Covariant	HR	95% Bootstrap BCa CI[b]	Bootstrap *P* value	HR	95% Bootstrap BCa CI	Bootstrap *P* value
Log_{10}(miR-224)	0.314	0.118–0.712	0.013	0.635	0.137–2.39	0.525
Gleason score (ordinal)	2.41	1.51–6.02	<0.001	2.054	0.975–4.91	0.022
Pathological stage (ordinal)	1.47	1.10–2.13	0.005	0.978	0.557–2.17	0.917
Preoperative PSA	1.13	1.02–1.32	0.001	1.076	0.866–1.28	0.143

[a] Adjusted for log_{10}miR-224 expression, Gleason score, pathological stage and preoperative PSA.
[b] BCa, bias corrected and accelerated.

(32); 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 1 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 I) *(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)*, *KLK10* (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 ($P < 0.001$) 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 ($r_s = -0.284$, $P = 0.015$) 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 Prueitt 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.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: European Union (European Social Fund – ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework Research Funding Program: THALIS-UOA (BIOPROMO).
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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