Combinations of Serum Prostate-Specific Antigen and Plasma Expression Levels of let-7c, miR-30c, miR-141, and miR-375 as Potential Better Diagnostic Biomarkers for Prostate Cancer

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In the current study, expression levels of let-7c, miR-30c, miR-141, and miR-375 in plasma from 59 prostate cancer (PC) patients with different clinicopathological characteristics and two groups of controls: 16 benign prostatic hyperplasia (BPH) samples and 11 young asymptomatic men (YAM) were analyzed to evaluate their diagnostic and prognostic value in comparison to prostate-specific antigen (PSA). miR-375 was significantly downregulated in 83.5% of patients compared to BPH controls and showed stronger diagnostic accuracy (area under the curve [AUC]=0.809, 95% CI: 0.697–0.922, $p=0.00016$) compared with PSA (AUC=0.710, 95% CI: 0.559–0.861, $p=0.013$). Expression levels of let-7c showed potential to distinguish PC patients from BPH controls with AUC=0.757, but the result did not reach significance. Better discriminating performance was observed when combinations of studied biomarkers were used. Sensitivity of 86.8% and specificity of 81.8% were reached when all biomarkers were combined (AUC=0.877) and YAM were used as calibrators. None of the studied microRNAs (miRNAs) showed correlation with clinicopathological characteristics. PSA levels were significantly correlated with the Gleason score, tumor stage, and lymph node metastasis with Spearman correlation coefficients: 0.612, 0.576, and 0.458. In conclusion, the combination of the studied circulating plasma miRNAs and serum PSA has the potential to be used as a noninvasive diagnostic biomarker for PC screening outperforming the PSA testing alone.

Introduction

PROSTATE CANCER (PC) is the most commonly diagnosed male malignancy and the second leading cause of male cancer-related death (Jemal *et al.*, 2010). It is a huge burden for the health system and despite advances in science, the etiology of this disease is not yet fully understood. The only estimated risk factors are age, ethnicity, family history, and diet (Plata Bello and Concepcion Masip, 2014). Hereditary factors account for 42% of PC risk (Lichtenstein *et al.*, 2000). At present, diagnosis of PC is derived from serum prostate-specific antigen (PSA) measurement, digital rectal examination (DRE), and histopathological evaluation of prostate needle biopsies (Gandellini *et al.*, 2010). PSA testing has a low specificity, and the optimal threshold for biopsy is unclear (Thompson *et al.*, 2003). In addition, PSA screening leads to over diagnosis and over treatment of indolent PCs (Dall'Era *et al.*, 2008). On the other hand, many

patients with cancer did not have elevated levels of PSA (Loeb and Catalona, 2008). Due to low specificity of PSA and low sensitivity of DRE, these tests have restricted diagnostic value (Backer, 1999). Current clinicopathological models also do not allow clinicians to accurately discern between lethal and indolent PC at an early stage, leading to anxiety for both clinicians and patients about choosing the best treatment course (Albertsen, 1998). Thus, there has been rapidly growing interest in alternative biomarkers such as microRNAs (miRNAs).

miRNAs are a class of 19–23 nucleotide long, endogenous noncoding RNA molecules that are frequently dysregulated in cancer. These miRNAs modulate the activity of transcriptome by binding to the 3'-untranslated regions of target mRNA sequences and leading to mRNA cleavage, decay, or inhibition of translation in various types of cancer (Bartel, 2004, 2009; Garzon *et al.*, 2006). Although miRNAs comprise ~3% of human encoded genes, more

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than 30% of mRNAs are regulated by miRNAs (Mahn *et al.*, 2011). They have been shown to play an important role in a wide range of biological and pathological processes, including cellular proliferation, differentiation, metastasis, immune response, metabolism, or apoptosis (Bartel, 2004, 2009; Brase *et al.*, 2010; Mahn *et al.*, 2011; Xu *et al.*, 2011). miRNA expression profiles are often tissue, developmental, and disease specific. Early work demonstrated that miRNA expression signatures are more useful than equivalent mRNA signatures and could accurately distinguish between different tumor types and are able to identify cancers of histologically uncertain origin (Lu *et al.*, 2005). Other useful characteristics of miRNAs for biomarker applications include their exceptional stability in various types of clinical samples, including formalin-fixed paraffin-embedded tissues (Mitchell, 2008), ease of quantitation using polymerase chain reaction (PCR)-based assays, and conservation between species (Selth *et al.*, 2012). miRNAs also show an ability to be sampled noninvasively. Circulating miRNAs are remarkably stable, resisting degradation by ribonucleases (Chen *et al.*, 2008; Mitchell, 2008).

Initially, miRNAs have been studied in normal and tumor tissue, in metastasis, and in PC cell lines (Porkka *et al.*, 2007; Ambs *et al.*, 2008; Bonci *et al.*, 2008; Lin *et al.*, 2008; Ozen *et al.*, 2008; Prueitt *et al.*, 2008; Yamakuchi *et al.*, 2008; De Vere White *et al.*, 2009; Ribas *et al.*, 2009; Coppola *et al.*, 2010; Ribas and Lupold, 2010; Schaefer *et al.*, 2010b; Spahn *et al.*, 2010; Catto *et al.*, 2011; Peng *et al.*, 2011; Takayama *et al.*, 2011; Watahiki *et al.*, 2011; Li *et al.*, 2012a; Martens-Uzunova *et al.*, 2012; Nadiminty *et al.*, 2012; Wach *et al.*, 2012). PC diagnostic and prognostic panels have been identified; however, they are partially overlapping or completely different in various studies. In PC, the efforts for tracing a reliable miRNA profile have proven inconclusive. Conflicting results between different datasets are possibly due to various study designs, underestimated treatments of the patients, methods of sample collection, presence of contaminating cells, and sensitivity and specificity of platforms used (Coppola *et al.*, 2010). In addition, the expression of miRNAs is dynamic and it is changed depending on the prostate carcinogenesis stage, type of therapy, and molecular pathway of every tumor.

To overcome the disadvantages of miRNA studies in tumors, researchers started to analyze miRNA expression in blood samples (plasma, serum) and even urine (Mitchell, 2008; Lodes *et al.*, 2009; Brase *et al.*, 2011; Mahn *et al.*, 2011; Moltzahn *et al.*, 2011; Yaman Agaoglu *et al.*, 2011; Zhang *et al.*, 2011; Bryant *et al.*, 2012; Chen *et al.*, 2012; Shen *et al.*, 2012; Srivastava *et al.*, 2013; Haj-Ahmad *et al.*, 2014).

In the current study, we analyzed the expression of miR-141, miR-375, let-7c, and miR-30c in the plasma of PC samples and controls consisting of benign prostatic hyperplasia (BPH) subjects and young asymptomatic men (YAM) to evaluate their diagnostic and prognostic value in Bulgarian patients. These miRNAs are extensively studied and their expression is correlated with PC development, metastasis, and other clinicopathological characteristics (Mitchell, 2008; Yaman Agaoglu *et al.*, 2011; Nadiminty *et al.*, 2012; Nguyen *et al.*, 2013), but still there is a need for more validation studies in plasma and serum.

The initial study of Mitchell (2008) has shown that miR-141, a miRNA involved in epithelial-mesenchymal transition (EMT), was elevated in the serum of metastatic PC

patients compared to healthy controls. The observation that elevated levels of this miRNA in serum and plasma were correlated with clinical progression, high Gleason score, and the development of metastasis in castration-resistant PC (mCRPC) was made in several different studies (Brase *et al.*, 2011; Gonzales *et al.*, 2011; Selth *et al.*, 2012; Bryant *et al.*, 2012; Nguyen *et al.*, 2013; Westermann *et al.*, 2014). However, a following study could not reproduce the significant difference in miR-141 levels and it was suggested that miR-141 levels were too low for reliable testing (Mahn *et al.*, 2011). In other studies it was found that plasma miRNA levels were similar in controls (healthy or BPH controls) and patients but could distinguish patients with metastasis from those with localized/local advanced disease (Yaman Agaoglu *et al.*, 2011; Zhang *et al.*, 2013). On the other hand, none of the analyzed miRNAs, including miR-141 in the study of Yaman Agaoglu *et al.* (2011), reached the power of PSA to discriminate metastatic PC from localized disease.

Similar to miR-141, circulating miR-375 levels have been correlated to the Gleason score, lymph node status, and distant metastasis (Brase *et al.*, 2011). The amount of miR-375 increases from low-risk through high-risk localized disease toward metastatic cancer (Bryant *et al.*, 2012; Selth *et al.*, 2012; Cheng *et al.*, 2013; Nguyen *et al.*, 2013).

Porkka *et al.* (2007) have shown that let-7c and miR-30c are with decreased expression in tissues of PC patients compared to tissues from BPH patients. Downregulation of miR-30c in PC tissues was observed in several other studies (Song *et al.*, 2013; Ling *et al.*, 2014; Ren *et al.*, 2014). Independently it was shown that let-7c and miR-30c in plasma are able to discriminate with high sensitivity and specificity the PC patients from BPH controls (Chen *et al.*, 2012). In addition, it was found that downregulation of let-7c and miR-30c in PC tissues and cells was associated with metastatic disease and androgen-dependent PC (Ren *et al.*, 2014). miR-30c expression in tissues showed also correlation with a higher Gleason score, advanced pathological stage, and biochemical recurrence (Ling *et al.*, 2014). It was estimated that downregulation of let-7c in PC specimens is inversely correlated with androgen receptor (AR) expression, whereas the expression of Lin28 (a repressor of let-7) is correlated positively with AR expression (Nadiminty *et al.*, 2012).

Materials and Methods

Participants and biological samples

After obtaining approval from the ethics review board (ethics committee) and informed consent from all study participants, blood samples from 59 PC patients and 16 BPH controls were drawn at the Clinic of Urology, Alexandrovskaya University Hospital, Medical University-Sofia. Blood samples from YAM were collected from 11 volunteers, mainly medical students. All blood samples were collected in EDTA tubes and processed within 1 h of collection. Blood was centrifuged to separate and collect plasma. Plasma samples were stored at -80°C until RNA extraction.

All subjects in the study were ethnic Bulgarians. BPH controls were matched to PC patients by age. Characteristics of PC patients and the two groups of controls are represented in Table 1.

TABLE 1. PARTICIPANTS' DEMOGRAPHIC AND CLINICAL CHARACTERISTICS

Characteristics	Patients (59)	BPH controls (16)	Controls YAM (11)
Median age	68	66	27
Range	51–83	55–88	21–35
Median PSA values	14.6	9.65	
Mean PSA values	19.43	11.24	
PSA values			
< 10 ng/mL	17	8	
10–30 ng/mL	32	8	
> 30 ng/mL	7	0	
Unknown	3	0	
Gleason score			
≤ 6	13		
7	24		
≥ 8	22		
T stage			
T1	9		
T2	29		
T3	18		
Unknown	3		
Lymph node metastasis			
N0	50		
N1	6		
Unknown	3		
Metastasis			
M0	55		
M1	2		
Unknown	2		
Age at diagnosis (PC or BPH)			
≤ 64	21	8	
> 64	38	8	

BPH, benign prostatic hyperplasia; PC, prostate cancer; PSA, prostate-specific antigen; YAM, young asymptomatic men.

RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was extracted from 200 µL plasma using the miRNeasy mini kit (Qiagen) following the manufacturer's instructions. The RNA was eluted in 40 µL nuclease-free water supplied with the kit. The concentrations and quality of RNA samples were evaluated by NanoDrop and Qubit. For reverse transcription reaction with the miScript II RT kit (Qiagen), 50 ng of each sample were used. Before use, every cDNA was diluted as recommended. Quantitative real-time PCR was done on the ABIPrism 7900HT (Applied Biosystems) with miScript Sybr Green PCR kit and miScript Primer Assays (Qiagen). Similar to other studies, RNU6B was used as reference control for normalization (Schaefer *et al.*, 2010a; Chen *et al.*, 2012; Gordanpour *et al.*, 2012).

Relative changes of gene expression levels of studied miRNAs were calculated by the $2^{-\Delta\Delta C_t}$ method.

Calibrators in the analysis were either BPH controls or YAM. Real-time experiments were performed in triplicates and the mean Ct values were calculated.

Statistical analysis

Statistical analysis was carried out with SPSS Statistics v. 20. The correlation between pairs of miRNA expression levels and PSA levels was evaluated using Pearson's cor-

relation coefficient. The Mann–Whitney U test and Spearman's correlation were used for comparison and estimation of correlations between miRNA expression levels and clinicopathological characteristics such as the Gleason score, tumor stage, lymph node metastasis, and age. Receiver operating curve (ROC) analysis was performed for evaluation of specificity and sensitivity of plasma miRNA expression levels for discriminating PC patients from controls. Diagnostic accuracy for combination of biomarkers was also determined by calculating weight coefficients for every biomarker obtaining the largest possible area under the curve (AUC) in ROC analysis. Calculation of coefficients was performed according Pepe and Thomson (2000). Two-tailed *p*-values were taken into account.

Results

Relative miRNA expression levels in plasma samples of PC patients were obtained by using RNU6B as a reference gene for normalization and BPH controls or YAM as calibrator samples. Relative quantification (RQ) values calculated by the $2^{-\Delta\Delta C_t}$ method were used for evaluation of expression in patients. RQ values between 0.500 and 1.999 show no significant difference in expression, values ≤ 0.499 show decreased expression, and values ≥ 2.00 show increased expression.

Slightly higher Ct values for the RNU6B reference control in the BPH group in comparison with the PC and YAM groups were observed, but the Mann–Whitney test showed there were no significant differences of Ct values between the studied groups.

All studied miRNAs, especially miR-141 and miR-375, showed downregulation in a high percent of patients in comparison with BPH controls (Fig. 1). Decreased expression of miR-141 and miR-375 was found in 71.43% and 83.05% of PC patients, respectively, compared to the BPH group (Table 2). When using YAM as calibrators, relative gene expression levels in a large proportion of patients did not reach statistically significant difference possibly due to the small sample size of the YAM controls. Results from the relative gene expression analysis for studied miRNAs when using BPH as calibrators are shown in Table 2.

It should be noted that expression of miR-141 was not detected in seven samples (four BPH and three PC samples) and the mean Ct (Cq) values among PC patients, BPH controls, and YAM were as follows: 30, 34, 32, respectively.

ROC was constructed to explore the potential value of analyzed miRNA expression levels as noninvasive diagnostic biomarkers for PC (Fig. 2). The miR-375 allowed most accurate discrimination (AUC=0.809, 95% CI: 0.697–0.922, $p=0.00016$) of cancer patients and BPH control subjects. At the optimal cutoff values of RQ, the sensitivity was 81.3% and specificity was 72.9%. miR-375 outperformed PSA serum levels (AUC=0.710, 95% CI: 0.559–0.861, $p=0.013$ with 76.8% sensitivity and 53.3% specificity at a cutoff value of 9.15 ng/mL) as the diagnostic biomarker in our study. We have chosen 9.15 ng/mL as the cutoff value for PSA because in the studied group of patients and BPH controls, only one person showed a PSA level under 4 ng/mL and the mean levels were high in the BPH group. At the cutoff value of 4 ng/mL the sensitivity was 100% but the specificity was barely 6.2%.

FIG. 1. Box plots representing plasma microRNAs (miRNAs) expression levels in prostate cancer (PC) patients and benign prostatic hyperplasia (BPH) controls. Expression levels of the miRNAs (scale of y axis: log 10) are normalized to RNU6B. BPH samples were used as calibrators.

let-7c could also discriminate PC patients from BPH controls with the following AUC=0.757 (95% CI: 0.622–0.893, $p=0.069$) and with 75% sensitivity and 61% specificity, but the result did not reach statistical significance. We obtained the following AUCs for miR-30c and miR-141: 0.630 (95% CI: 0.475–0.786, $p=0.079$) and 0.510 (95% CI: 0.296–0.723, $p=0.91$), respectively. At the optimal cutoff value, the sensitivity and specificity were 62.5% and 42.4% for miR-30c and 50% and 71.2% for miR-141.

Constructed ROC to determine the diagnostic accuracy of these four miRNAs in differentiating PC from BPH and YAM, when using the last as calibrator samples, had smaller areas under the curve: let-7c, 0.626 (95% CI: 0.498–0.755, $p=0.626$); miR-30c, 0.586 (95% CI: 0.455–0.717, $p=0.204$); miR-141, 0.567 (95% CI: 0.412–0.722, $p=0.353$); and miR-375, 0.711 (95% CI: 0.595–0.826, $p=0.02$). At the optimal cutoff value, the sensitivity and specificity were let-7c, 63% and 61%; miR-30c, 55.6% and 54.2%; miR-141, 56.5% and 57.1%; miR-375, 77.8% and 62.7%.

Expression levels of miR-375 in our study proved to be the most reliable noninvasive biomarker for discriminating PC patients from the two groups of controls.

Some combinations of the studied miRNAs and PSA improved the diagnostic accuracy in ROC analysis. When using BPH as calibrator samples, the best combination of

biomarkers with the highest sensitivity was between miR-30c, miR-141, miR-375, and PSA, but the specificity was lower compared with miR-375 alone. The largest AUC was observed when combining let-7c, miR-141, miR-375, and PSA. Unfortunately, the combination of all five biomarkers did not outperform miR-375. Selected results from the multimarker ROC analysis when BPH samples were used as calibrators are shown in Table 3 and Figure 3A.

Similar patterns were observed when multimarker ROC analysis was performed when YAM were used as calibrators (Table 4 and Fig. 3B). In this analysis, the combination of all 5 biomarkers significantly outperformed miR-375 and the largest AUC was obtained: 0.877.

In addition, we investigated whether any of the studied miRNAs were significantly correlated with clinicopathological characteristics like the Gleason score, tumor stage, and development of lymph node metastasis and then we compared the results obtained for PSA. We were not able to check if the expression levels of miRNAs in plasma were correlated with the risk of distant metastasis development because we had only two patients with metastasis. In our study, only PSA showed a statistically significant strong correlation with the Gleason score, tumor stage, and lymph node metastasis with Spearman correlation coefficients: 0.612 ($p=5.31 \times 10^{-7}$); 0.576 ($p=4.21 \times 10^{-6}$); and 0.458

TABLE 2. RESULTS FROM RELATIVE MICRORNA EXPRESSION ANALYSIS IN PLASMA SAMPLES OF PROSTATE CANCER PATIENTS AND BENIGN PROSTATIC HYPERPLASIA CONTROLS AS CALIBRATORS

	let-7c	miR-30c	miR-141	miR-375
<i>Results: PC patients vs. BPH controls</i>				
	<i>Number of PC patients (%)</i>			
Without significant difference in expression	10 (16.95)	13 (22.03)	11 (19.64)	1 (1.7)
Decreased expression	40 (67.8)	35 (59.32)	40 (71.43)	49 (83.05)
Increased expression	9 (15.25)	11 (18.64)	5 (8.93)	9 (15.25)

FIG. 2. Receiver operating curve (ROC) curve analysis by using four miRNAs and prostate-specific antigen (PSA) to differentiate PC ($n=59$) from controls without cancer: (A) when BPH group ($n=16$) was used as calibrator and (B) when young asymptomatic men (YAM) group ($n=11$) was used as a calibrator.

($p=4.34 \times 10^{-4}$). The observed correlations were also confirmed by the Mann–Whitney U test. We performed ROC analysis to evaluate the serum PSA values as predictors for the presence of lymph node metastasis and obtained the following results: AUC=0.960 (95% CI: 0.908–1.00, $p=0.001$), and at the cutoff value of 27.1 ng/mL, the sensitivity was 100% and the specificity was 90%.

To check if the expression levels of the studied miRNAs are correlated with age, we performed the Spearman test,

appropriate for not normally distributed data. We did not observe statistically significant correlation between age and expression levels of the studied miRNAs within the three groups (PC patients, BPH, and YAM) or as a whole.

The expression levels of let-7c showed statistically significant correlation with the expression levels of miR-30c, miR-141, and miR-375 with the following Pearson's correlation coefficients: 0.650 ($p=2.84 \times 10^{-10}$), 0.447 ($p=5.7 \times 10^{-5}$), and 0.266 ($p=0.021$). Similar correlation

TABLE 3. SELECTED RESULTS FROM MULTIMARKER RECEIVER OPERATING CURVE ANALYSIS WHEN BPH SAMPLES ARE USED AS CALIBRATORS

Combination of biomarkers	AUC (95% CI)	p-Value	Sensitivity (%)	Specificity (%)
let-7c and miR-141	0.753 (0.598–0.908)	0.002	79.7	81.2
miR-141 and miR-375	0.695 (0.510–0.880)	0.017	74.6	75
let-7c and PSA	0.714 (0.560–0.868)	0.012	69.1	60
miR-30c and PSA	0.702 (0.546–0.858)	0.017	65.5	60
miR-141 and PSA	0.699 (0.551–0.846)	0.019	72.7	53.3
miR-375 and PSA	0.833 (0.727–0.938)	8.5×10^{-5}	85.5	66.7
let-7c, miR-30c, and miR-141	0.753 (0.598–0.908)	0.002	79.7	81.2
let-7c, miR-141, and miR-375	0.816 (0.688–0.943)	1.2×10^{-4}	78	87.5
let-7c, miR-375, and PSA	0.838 (0.732–0.943)	7×10^{-5}	70.9	80
let-7c, miR-30c, and PSA	0.719 (0.567–0.870)	0.01	72.7	53.3
let-7c, miR-141, and PSA	0.705 (0.554–0.857)	0.015	65.5	60
miR-30c, miR-375, and PSA	0.833 (0.725–0.940)	8.6×10^{-5}	70.9	80
miR-30c, miR-141, and PSA	0.699 (0.552–0.847)	0.019	65.5	60
miR-30c, miR-141, and miR-375	0.814 (0.686–0.941)	1.3×10^{-4}	76.3	87.5
let-7c, miR-30c, miR-375	0.662 (0.487–0.837)	0.048	71.2	62.5
miR-141, miR-375, PSA	0.834 (0.724–0.944)	8.0×10^{-5}	70.9	73.3
let-7c, miR-30c, miR-141, and miR-375	0.781 (0.635–0.927)	0.01	81.4	81.2
let-7c, miR-141, miR-375, and PSA	0.839 (0.733–0.944)	6.3×10^{-5}	92.7	66.7
let-7c, miR-30c, miR-375, and PSA	0.839 (0.735–0.943)	6.3×10^{-5}	92.7	66.7
let-7c, miR-30c, miR-141, and PSA	0.777 (0.657–0.897)	0.001	80	60
miR-30c, miR-141, miR-375, and PSA	0.836 (0.726–0.947)	7.1×10^{-5}	94.5	66.7
let-7c, miR-30c, miR-141, miR-375, and PSA	0.782 (0.664–0.899)	0.001	63.6	73.3

AUC, area under the curve.

FIG. 3. Multimarker ROC: (A) BPH samples used as calibrators and (B) YAM samples used as calibrators.

was observed for miR-141 and miR-30c (Pearson correlation coefficient 0.371, $p=0.001$).

Discussion

PC was the first cancer type to be used as a disease model for the establishment of circulating miRNAs' potential as blood-based biomarkers (Mitchell, 2008). Since the work of

Mitchell and colleagues, many studies examined circulating miRNAs and have shown that they are correlated with PC development and progression. In several studies, miR-141, miR-375, let-7c, and miR-30c have been analyzed in plasma and/or serum and have shown great potential (Brase *et al.*, 2011; Gonzales *et al.*, 2011; Mahn *et al.*, 2011; Moltzahn *et al.*, 2011; Yaman Agaoglu *et al.*, 2011; Bryant *et al.*, 2012; Chen *et al.*, 2012; Selth *et al.*, 2012; Nguyen *et al.*, 2013).

TABLE 4. SELECTED RESULTS FROM MULTIMARKER RECEIVER OPERATING CURVE CURVE ANALYSIS WHEN YOUNG ASYMPTOMATIC MEN SAMPLES ARE USED AS CALIBRATORS

Combination of biomarkers	AUC (95% CI)	p-Value	Sensitivity (%)	Specificity (%)
miR-375 and PSA	0.823 (0.709–0.936)	1.3×10^{-4}	85.7	73.3
let-7c and miR-375	0.701 (0.575–0.828)	0.003	76.3	66.7
let-7c and PSA	0.786 (0.669–0.903)	0.001	73.2	80
miR-30c and miR-375	0.693 (0.564–0.822)	0.004	74.6	70.4
miR-30c and PSA	0.714 (0.562–0.867)	0.011	76.8	53.3
miR-141 and miR-375	0.747 (0.636–0.858)	0.001	76.8	69.6
miR-141 and PSA	0.748 (0.585–0.911)	0.01	62.3	63.6
let-7c, miR-30c, and miR-375	0.702 (0.574–0.830)	0.003	79.7	63
let-7c, miR-30c, and PSA	0.795 (0.680–0.910)	0.0005	78.6	80
let-7c, miR-141, and miR-375	0.714 (0.579–0.848)	0.003	73.2	73.9
let-7c, miR-375, and PSA	0.815 (0.696–0.935)	0.0002	78.6	80
let-7c, miR-141, and PSA	0.811 (0.676–0.947)	0.001	83	72.7
miR-30c, miR-375, PSA	0.824 (0.714–0.934)	0.0001	71.4	80
miR-30c, miR-141, and miR-375	0.688 (0.543–0.832)	0.009	82.1	60.9
miR-30c, miR-141, and PSA	0.768 (0.620–0.917)	0.005	69.8	63.6
let-7c, miR-30c, miR-141, and miR-375	0.769 (0.650–0.889)	0.0002	76.8	78.3
let-7c, miR-141, miR-375, and PSA	0.875 (0.779–0.971)	0.0001	86.8	81.8
let-7c, miR-30c, miR-375, and PSA	0.815 (0.690–0.940)	0.0002	82.1	80
let-7c, miR-30c, miR-141, and PSA	0.835 (0.726–0.945)	0.001	83	72.7
miR-30c, miR-141, miR-375, and PSA	0.858 (0.750–0.965)	0.00021	88.7	72.7
let-7c, miR-30c, miR-141, miR-375, and PSA	0.877 (0.782–0.971)	9×10^{-5}	86.8	81.8

In our study, the Ct values for miR-141 were high, while expression was not detected in several samples. In consistency to other studies, the levels of miR-141 were similar in patients and YAM (Mahn *et al.*, 2011; Yaman Agaoglu *et al.*, 2011), but this is in contrast to the findings of Mitchell (2008). The discrepancies between studies may be due to differences in miRNA abundance between serum and plasma, different methods used for analysis (for example, inclusion of preamplification step between reverse transcription and real-time PCR to increase the amount of low-abundant miRNAs) and sample size. Another possible explanation is that Mitchell and coworkers compared miRNA levels between metastatic PC and healthy controls. We were not able to check the correlation of miR-141 with metastasis development due to the small number of such patients. In addition, no correlations with other clinicopathological characteristics (Gleason score, tumor stage, lymph node affliction) were observed, due probably to the small sample size and the very low levels in plasma of this miRNA. ROC analysis in our study proved that miR-141 levels could not reliably discriminate patients from BPH and YAM groups (Fig. 2).

The levels of miR-375 had been compared between different groups of PC patients or between mCRPC cases and healthy controls and it was confirmed that circulating miR-375 levels were elevated in mCRPC in comparison to healthy controls or patients with localized PC (Brase *et al.*, 2011; Bryant *et al.*, 2012; Cheng *et al.*, 2013; Nguyen *et al.*, 2013). In the current study, we have compared plasma expression levels of this miRNA in PC patients and two groups of controls (BPH and YAM). We have found significantly decreased expression of miR-375 in 83.05% of PC patients compared to BPH controls (Table 2). In comparison with YAM, 45.76% of the patients showed decreased expression. The discrepancy could be possibly attributed to the composition of the investigated groups. In our group of patients there were only two with metastasis and six with positive lymph nodes and this may be a reason for the observed decreased expression. The expression of miR-375 is downregulated also in gastric cancer (Ding *et al.*, 2010; Tsukamoto *et al.*, 2010), head and neck squamous cell carcinoma (Avissar *et al.*, 2009; Harris *et al.*, 2012), squamous cell carcinoma of the esophagus (Mathe *et al.*, 2009), oral tumors (Jung *et al.*, 2013), nonsmall lung cancer (Li *et al.*, 2012b), hepatocellular tumors (Ladeiro *et al.*, 2008), pancreatic cancer (Zhou *et al.*, 2012), and colorectal cancer (Dai *et al.*, 2012; Faltejiskova *et al.*, 2012). In breast cancer, miR-375 is overexpressed in ER α -positive breast cell lines and it is a key driver of their proliferation (de Souza Rocha Simonini *et al.*, 2010). Since the expression of estrogen receptor is elevated in PC development, upregulation of miR-375 could assist tumor progression. In the study of Jung *et al.*, it was found that miR-375 acts as a tumor suppressor in oral cancer and reduces the expression of CIP2A, resulting in a decrease of MYC protein levels and leading to reduced proliferation, colony formation, migration, and invasion (Jung *et al.*, 2013). In PC, MYC also has shown increased expression during tumorigenesis (Ellwood-Yen *et al.*, 2003), in androgen-dependent cancer, and in CRPC (Jenkins *et al.*, 1997; Hawksworth *et al.*, 2010). The mechanism of MYC upregulation in PC is yet not completely revealed, but it is possible that miR-375 plays a role in its regulation as in oral cancer.

ROC analysis in our study showed that downregulation of miR-375 in plasma is the most accurate diagnostic biomarker and can distinguish PC patients from controls with high sensitivity and specificity (Fig. 2). Our study demonstrated that miR-375 levels in plasma could be used not only as a prognostic but also as a diagnostic biomarker. However, we were not able to find statistically significant correlation of miR-375 with clinicopathological characteristics.

The observed downregulation of miR-375 in the current study could be explained not only with the small number of patients with distant metastasis and positive lymph nodes but also with the possibility for the presence of different comorbidities as diabetes, prostatitis, different pathophysiological processes, and androgen deprivation therapy. In addition to the role of miR-375 in various types of cancer, it has also a critical role in the regulation of key pathophysiological mechanisms as glucose metabolism (Lynn, 2009), hepatitis B virus infections (Li *et al.*, 2010), participates in multiple allergic diseases induced by interleukin-13 (IL-13) (Lu *et al.*, 2012), and in the development of diabetes (Poy *et al.*, 2004; Tang *et al.*, 2008; Erener *et al.*, 2013). It was recently found that AR levels are negatively correlated with the methylation-mediated transcriptional repression of miR-375 in human PC cells (Chu *et al.*, 2014). In AR-negative PCs, the level of miR-375 has been low due to the hypermethylation of its promoter related to high DNA methyltransferases activity. In AR-positive PC, the opposite was observed (Chu *et al.*, 2014).

The other miRNAs studied by us are let-7c and miR-30c. Members of let-7 family were found to be downregulated in various types of cancer with few exceptions. Particularly in PC (Chen *et al.*, 2012; Schubert *et al.*, 2013), breast cancer (Cava *et al.*, 2014), ovarian cancer (Dahiya *et al.*, 2008), nonsmall cell lung cancer (Zhao *et al.*, 2014) and in hepatocellular carcinoma (Li *et al.*, 2013), let-7c is downregulated. The functionality of let-7 has been shown to target oncogenes involved in cell cycle regulation, cell migration, proliferation, differentiation, and EMT progression (Johnson *et al.*, 2005; Kumar *et al.*, 2007; Lee and Dutta, 2007; Dong *et al.*, 2010; Kong *et al.*, 2012).

Expression levels of miR-30c are frequently increased in different tumors, including ovarian cancer (Lee *et al.*, 2012), mesothelioma (Busacca *et al.*, 2010), primary cutaneous anaplastic large cell lymphoma (Benner *et al.*, 2012), but are downregulated in breast cancer (Tanic *et al.*, 2012), renal cell carcinoma (Heinzelmann *et al.*, 2011), prostate cancer (Porkka *et al.*, 2007; Chen *et al.*, 2012; Song *et al.*, 2013; Ling *et al.*, 2014; Ren *et al.*, 2014), and bladder cancer (Wang *et al.*, 2010).

In our study, in consistency with other studies, let-7c and miR-30 were downregulated in the majority of patients compared to BPH samples used as calibrators. In contrast, PC patients were equally distributed in groups (with increased, with decreased, or without difference) according to their expression when compared to YAM samples used as calibrators. Therefore, in our study, when YAM were used as calibrators in ROC analysis for all studied miRNAs, we included BPH samples to see if discrimination between PC versus the BPH and YAM group will be improved. ROC analysis when BPH samples were used as calibrators showed that the let-7c levels in plasma had a potential to be used as diagnostic biomarkers for discriminating PC patients from BPH controls with AUC=0.757 and sensitivity of 75%

and specificity of 61% (Fig. 2). The specificity was higher and the sensitivity was similar to those obtained from the ROC analysis of PSA. Similar AUC (0.784) for let-7c was obtained by Chen *et al.* (2012). When YAM were included as calibrators, a smaller AUC (0.626) was derived in ROC analysis for discriminating PC patients from BPH and YAM subjects. Chen and colleagues had a larger AUC (0.775), but their group of healthy controls consisted of older men without BPH and with a mean age of 72 years. Furthermore, they have analyzed a larger group. This could also be a reason for the smaller AUC produced by us in ROC analysis. Neither let-7c nor miR-30c showed correlation with clinicopathological characteristics in our study.

After its approval by the U.S. Food and Drug Administration (FDA) in 1986, the PSA test revolutionized the PC screening and diagnosis landscape. Nonetheless, there are inherent limitations to using the PSA test for PC screening. First, the test may give false-positive or false-negative results. Most men with an elevated PSA level (above 4.0 ng/mL) are not found to have PC; only about 25% of men who undergo a prostate biopsy due to an elevated PSA level actually have PC. Conversely, a negative result may give false assurances that PC is not detected, when in fact a cancer may exist (NCI, 2014).

Overall, the performance of PSA testing as a screening tool for PC is known to be variable. Depending on the PSA cutoff values applied, the specificity and sensitivity of PSA range from 20% to 40% and 70% to 90%, respectively (Prensner *et al.*, 2012). The AUC of the ROC analysis is between 0.55 and 0.70 for the ability of PSA to identify PC (Prensner *et al.*, 2012). AUC values close to 1 belong to biomarkers that are perfect discriminators. Due to a high false-positive rate, PSA screening for PC demonstrates a positive predictive value of only 25–40% (Schroder *et al.*, 2008). One of the main reasons for the low specificity would be that PSA may be elevated as a result of various non-cancerous conditions such as infections, trauma, and BPH (Barry, 2001; Lilja *et al.*, 2008). In addition, around 15–20% from men with low levels of PSA (<4.0 ng/mL) have PC and around 15% of them have high Gleason score values (Thompson *et al.*, 2004).

The results from our study are close to those published in the literature. The AUC was 0.710 (95% CI: 0.559–0.861, $p=0.013$), sensitivity was 76.8%, and specificity was 53.3% at a cutoff value 9.15 ng/mL (Fig. 2). At the cutoff value of 4 ng/mL, the sensitivity was 100%, but the specificity was barely 6.2%. In contrast from the other studies for evaluation of specificity and sensitivity of PSA, we used a higher cutoff value since in our group of BPH samples, the mean PSA levels were 11.24 and there was only one BPH sample with the PSA level under 4 ng/mL.

Several studies have shown that monitoring the PSA values could be used for evaluating the risk for development of aggressive cancer (advanced tumor stage, high Gleason score, and metastasis) in patients with PC (Antenor *et al.*, 2005; Kundu *et al.*, 2007; Ulmert *et al.*, 2008; Vickers *et al.*, 2010; Corcoran *et al.*, 2012). PSA levels together with Gleason score and tumor stage are used to calculate the risk for development of aggressive PC, for occurrence of biochemical relapse after radical prostatectomy in different classifiers as D'Amico, CAPRA score (D'Amico *et al.*, 1998; Patel *et al.*, 2007). In line with the studies that have

shown correlation of PSA with clinicopathological characteristics, our study shows statistically significant correlation of PSA with Gleason score, tumor stage, and lymph node metastasis with Spearman correlation coefficients: 0.612 ($p=5.31 \times 10^{-7}$); 0.576 ($p=4.21 \times 10^{-6}$); and 0.458 ($p=4.34 \times 10^{-4}$). PSA levels at diagnosis could predict the presence of lymph node metastasis with 100% sensitivity and 90% specificity when 27.1 ng/mL was used as the cutoff value.

ROC analysis was used to further evaluate the joint diagnostic value of the four studied miRNAs and PSA. More powerful diagnostic values were observed when combining miRNAs with or without PSA. The best discrimination between PC patients and the two groups of controls with 86.8% sensitivity and 81.8% specificity was observed when all biomarkers were combined and YAM were used as calibrator samples (Fig. 3). The obtained AUC was 0.877. When BPH samples were used as calibrators, several combinations could make better discrimination between groups in comparison with single biomarkers. When all four miRNAs were combined, AUC was 0.781 and this set could discriminate PC patients from BPH controls with 81.4% sensitivity and 81.2% specificity. Another reliable combination was between miR-30c, miR-141, miR-375, and PSA with AUC=0.836 and sensitivity 94.5% and specificity 66.7%.

To conclude, the use of panels consisting of miRNAs and PSA has advantages over single miRNAs or PSA because different miRNAs have aberrant expression in different subtypes of cancer and PSA is not cancer specific.

Several specific issues and limitations should be pointed out in our study. For the first time, we report downregulation of miR-141 and miR-375 in plasma of PC patients compared to BPH samples. The plausible reasons for this could be heterogeneous by the disease stage group, with only two patients with distant metastasis included. In the other published studies, the comparisons of expression levels of circulating miR-141 and miR-375 were made between PC patients with or without metastasis or between patients with metastasis and healthy controls to differentiate patients at a high risk. In contrast, we compared the expression levels between all patients and controls. Another explanation for the miR-375 results could be that this miRNA plays a role in several pathophysiological processes and is associated not only with cancer but also with diabetes, allergic conditions, and inflammations. Unfortunately, we did not have sufficient data for the comorbidities of all analyzed patients to test this hypothesis as well. In addition, the expression levels of plasma miR-375 and its CpG methylation in the promoter region could be ethnically different, as this was recently demonstrated in a study of type 2 diabetes (Chang *et al.*, 2014). It was also shown that the AR is negatively correlated with the methylation-mediated transcriptional repression of miR-375 in human PC cells (Chu *et al.*, 2014). Thus, any prior hormone treatment of the patients affecting the level of AR would also have influence on the level of miR-375 in human PC cells, hence on the circulating miR-375 in plasma.

For miR-141, we have obtained higher Ct values in comparison with the other studied miRNAs, probably due to the lower level of this miRNA in plasma compared with serum and as we have not used a preamplification step.

The second issue is that we did not observe correlation of the studied miRNAs with the clinicopathological characteristics. Such correlations were observed only for PSA. Probably the miRNA expression was not so dramatically influenced by the Gleason score, tumor stage, and the development of lymph node metastasis as serum PSA levels in the investigated samples. Another limitation is the comparatively small size of the investigated groups. A future larger independent study is needed for making more definitive conclusions on this correlation.

Conclusion

We have shown that the expression level of miR-375 in plasma outperformed serum PSA levels as a diagnostic biomarker for PC. let-7c miRNA also showed potential for discrimination of PC patients and controls. Combinations of let-7c, miR-30c, miR-141, miR-375, and PSA obtained even better discrimination and could be more useful than PSA alone as noninvasive diagnostic biomarkers for screening of PC. PSA levels showed correlation with the Gleason score, tumor stage, and lymph node metastasis, but such correlations were not observed for the studied miRNAs. Because of the still conflicting data in the literature, standardized methodologies and larger sample sets are crucial for exploring the clinical potential of circulating miRNAs.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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