Serum microRNAs as Biomarkers in Patients Undergoing Prostate Biopsy: Results from a Prospective Multi-Center Study

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Abstract. *Background/Aim: Increased levels of microRNAs in serum/plasma have been identified in various malignancies. We aimed to investigate serum levels of miR-26a-1 and miR-141 in patients undergoing prostate biopsy clinical suspicious for prostate cancer (PCA) in a prospective multi-center study. Patients and Methods: Pre-biopsy serum samples of 170 patients were collected in three different study Centres. Serum RNA was isolated, and microRNA lev-els were quantified using real-time PCR. Relative miR-26a -1 and miR-141 levels were determined using RNU1-4 and SNORD43 as reference genes. Results: After exclusion of pa-tients with metastatic prostate cancer (n=9) and isolation failures (n=28), 133 patients (prostate cancer n=54, non-malignant n=79) were used for further analysis. The levels of miR-26a-1 and miR-141 were similar in patients with positive and negative biopsies. We observed a significant increase of miR-141 in patients with higher Gleason Score. Conclusion: The analysis of circulating microRNAs does not seem to help identify patients with cancer undergoing prostate biopsy. However, their levels may be useful to identify patients with high-risk prostate cancer.*

Micro RNAs are small single-stranded endogenous non-coding RNA molecules of approximately 23 nucleotides in length. They are involved in the regulation of basic cellular processes like differentiation, proliferation and apoptosis, and may have oncogenic or tumor suppressive roles (1-3). Specific microRNA expression profiles in tissue of various human malignancies have been discovered (4-7). Few years ago, the existence of circulating microRNAs in serum and plasma was demonstrated (8, 9). Subsequently various researchers highlighted the potential of these circulating microRNAs as non-invasive diagnostic and/or prognostic cancer biomarkers (10-15).

Prostate cancer (PCA) is suspected in patients with increased serum prostate specific antigen (PSA) levels or abnormal digital rectal examination. Especially PSA is nowadays the most important parameter for indicating PCA in biopsies. However, prostate needle biopsy is limited by excessive-negative biopsies, false-negative biopsies, and over-diagnosis of clinically-insignificant cancers (16-21). Thus, improved biomarkers are warranted to reduce unnecessary biopsies and to discriminate between aggressive and slow-growing cancers.

We have earlier reported that tumor-associated miR-26a-1 levels in serum allow for discrimination of PCA and benign prostate hyperplasia patients, especially in a subset of patients with increased PSA levels; thus miR-26a-1 could be of potential diagnostic relevance (22). Serum levels of miR-141 were increased in patients with aggressive PCA (23-26), indicating its prognostic potential. Former studies investigating on the relevance of microRNAs as adjunct diagnostic/prognostic tool compared serum of patients with PCA undergoing radical prostatectomy with healthy controls or patients with benign prostate hyperplasia. Thereby, these analyses did not represent a real-life setting, in which patients are scheduled for prostate biopsy to confirm/exclude the clinical suspect of PCA, and the achieved results may be biased. We, thus, investigated miR-26a-1 and miR-141 in serum of patients undergoing prostate biopsy for suspicion of clinically-localized prostate cancer.

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Key Words: Prostate cancer, biomarker, serum, microRNA, miR-141, miR-26a-1.

Patients and Methods

Patients and sample processing. We prospectively collected 170 serum samples from patients scheduled for prostate biopsy between August 2007 and December 2013. The recruitment of patients and collection of serum samples was performed at the Ambulatory Urological Center Bonn (MVZ), and the Departments of Urology at the University Hospital Bonn (UKB) and Munster (UKM). All patients gave written informed consent for collection of serum samples. The study was approved at the Ethics' Committee of the University of Bonn (approval number 141/11).

The indication for prostate biopsy was based on a suspicious digital-rectal examination or PSA elevation (>4 ng/ml). The following clinicopathological information were recorded: age, abnormalities in the transrectal ultrasonography or digital rectal examination, PSA and free PSA (fPSA), number of previous prostate biopsies, family history for prostate cancer, Gleason Score and clinical stage.

Blood was obtained according to standard operating procedures: blood was with-drawn in a Serum S-Monovette (Sarstedt, Nürnberg, Germany) with clotting activator. Centri-fugation was performed within 2 h and serum was subsequently separated and stored in cryotubes at –80˚C in the Biobanks of the Universities Bonn and Münster until use.

RNA extraction and microRNA quantification. The detailed description of RNA extraction and microRNA quantification is provided in a MIQE-compliant report in the Supplementary Material. In brief, isolation of total RNA was carried out using the Ambion MirVana Paris-Kit (Life Technologies, Darmstadt, Germany). In order to control sample-to-sample variation and isolation efficiency, the protocol was modified by the addition of 25 fmol of synthetic *Caenorhabditis elegans* miRNA (cel-miR-39) into 400 μl serum at the beginning of the isolation process as described by Mitchel *et al*. (9). The final elution volume was 50 μl.

Reverse Transcription (using 15 μl of isolated RNA) and quantitative real-time PCR reaction was performed with miScript RT Kit and miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany) according to the manufactures protocol. MiScript PCR Assays (cel-miR-39, RNU1-4, SNORD43, mir-26a-1, mir-141) were purchased from Qiagen. All PCR reactions were car-ried out on an ABIPrism 7900 HT in triplicates with each 10μl reaction volume. Each PCR included negative controls (water blanks, genomic DNA, no-reverse transcription sample) and positive controls (serial-dilution of LNCaP RNA).

Data analysis and statistical methods. PCR data were analyzed with the SDS Relative Quantification Software v2.4 and the RQ Manager v1.2.1; relative quantification was performed using Data Assist v3.0 (all software packages: Applied Biosystems, Darmstadt, Germany). Relative circulating microRNA levels were calculated using the 2-ΔΔCT formula with *RNU1-4* and *SNORD34* as references genes; we earlier demonstrated a combination of both as most stable reference gene for quantification of serum microRNAs in patients with prostate cancer (27).

Statistical analysis was performed with SPSS Statistics v21 (SPSS, Chicago, IL, USA). Differences of circulating micro RNA levels between patients with negative and positive bi-opsy were analysed using Kolmogorov-Smirnov-test and Mann-Whitney-*U*-test. Correlation of micro RNA levels and PSA levels was performed with Spearman rank correlation test. Receiver operating characteristics (ROC) curves were calculated to determine the potential of micro RNAs to discriminate between cancer and benign histology. Clinicopathological parameters and microRNA levels were correlated using the Mann-Whitney-*U* or the Kruskal-Wallis-test as appropriate.

Table I. *Clinicopathological parameters of the studied population*.

	Control (n=79)	PCA (n=54)	*p*-Value
Age (years)			<0.001
Range	43-79	47-79	
Median	63	70	
Number of biopsies			0.093
First biopsy	54 (68.4%)	44 (81.5%)	
Second biopsy	25 (31.6%)	10 (18.5%)	
DRE			0.005
Suspect	28 (35.4%)	33 (61.1%)	
Normal	44 (55.7%)	18 (33.3%)	
Missing	7 (8.9%)	3 (5.6%)	
TRUS			<0.001
Suspect	25 (31.6)	37 (68.5%)	
Normal	48 (60.8%)	15 (27.8%)	
Missing	6 (7.6)	2 (3.7)	
Prostate volume (ml)			0.024
Range	13-120	17-80	
Median	45	37	
Missing	7	1	
PSA (ng/ml)			0.357
Range	0.99-72.7	0.86-36.69	
Median	7.3	8.5	
fPSA (%)			0.357
Range	<1-30	2-28	
Median	13.9	12.3	
Missing	12	12	
Gleason Score			
6		21 (38.9%)	
7		24 (44.4%)	
8		4 (7.4%)	
9		5 (9.3%)	
Clinical tumor stage			
cT1c		21 (38.9%)	
cT2		25 (46.3%)	
cT3		4 (7.4%)	
cT4		1 (1.9%)	
Missing		3 (5.6%)	

PCA, Prostate cancer; DRE, digital rectal examination; TRUS, transrectal ultrasonography; PSA, prostate-specific antigene; fPSA, free prostate specific antigen. Prostate volume was measured by TRUS.

Results

Patients and tumor characteristics. We collected serum samples from 170 patients who underwent prostate biopsy. Nine samples from patients with metastatic PCA were excluded because biopsy was solely performed for histological confirmation of the clinical diagnosis. In addition, serum RNA isolation failed in 28 cases, and these

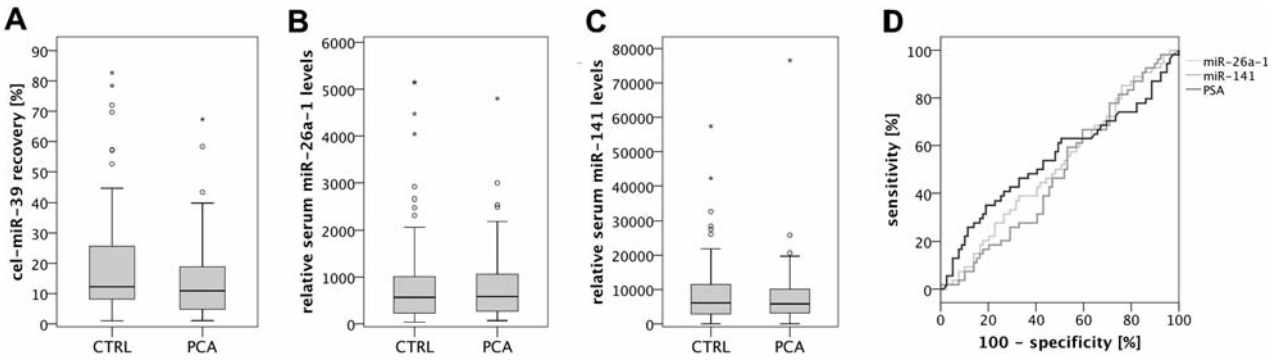

Figure 1. *The boxplots demonstrate (A) the RNA isolation efficiency, which was determined as recovery of cel-miR-39. The serum levels of (B) miR-26a-1 and (C) miR-141 were quantified relatively to the amount of circulating RNU1-4 and SNORD43, using the 2-delta-delta-Ct-formula; serum microRNA levels were similar in patients with prostate cancer and control subjects. Receiver operator characteristic analysis (D) shows that neither miR-26a-1, miR-141 nor PSA levels are helpful to distinguish patients with positive and negative prostate biopsy.*

samples were also excluded from further analysis. Among the remaining 133 patients, PCA was diagnosed in 54 patients. The samples numbers from each Center were: UKB n=43 (PCA n=18, non-malignant n=25), UKM n=77 (PCA n=32, non-malignant n=45) and MVZ n=13 (PCA n=4, non-malignant n=9). Clinicopathological information of the final study cohort is summarized in Table I.

RNA isolation efficiency. The quantification of cel-miR-39 enabled us to control sample-to-sample variation and isolation efficiency. In 28 samples the recovery rate was below 1% and 4 over 100%; these samples were regarded as isolation failures, and thus omitted from our analyses. The mean recovery rate of the remaining 133 samples was 17.8% (range=1%-100%). The mean RNA recovery was similar in patients with positive (mean=14.8%, inter-quartile range (IQR) 15.1%) and negative (mean 19.8%, IQR 18.3%) biopsy (p=0.107). See Figure 1A. The diverging RNA isolation efficiencies encouraged to use the small nucleolar RNAs SNORD43 and RNU1-4 as a reference gene for a relative quantification approach, which is not biased by the RNA recovery rate (27).

Serum miR-26a-1 and miR-141 as diagnostic biomarker. Serum microRNA levels were not increased in patients with positive prostate biopsy (Figure 1B-D): mean miR-26a-1 levels were 860.9 (IQR 796.3) in PCA patients and 913.1 (IQR 800.4) in patients without malignancy (p=0.717). Serum miR-141 levels were 8518.7 (IQR 6997.7) in PCA patients and 9099.3 (IQR 8863.9) in controls (p=0.840). The failure of both microRNAs as diagnostic biomarker was confirmed by ROC analyses: the area under the curve (AUC) was 0.519 (95% confidence interval (CI) 0.419-0.618) for miR-26a-1 and 0.49 (95% CI 0.391-0.588) for miR-141. Notably, PSA (AUC 0.547; 95% CI 0.443-0.651) and %fPSA (AUC 0.447; 95% CI 0.334-0.561) also failed to provide any diagnostic benefit in our cohort. We also carried out the analyses for the UKM and UKB cohorts separately (analyses for MVZ patients only were not performed due to the small amount of samples); serum microRNA levels of patients with positive and negative biopsies were not different within these sub-groups (p>0.3).

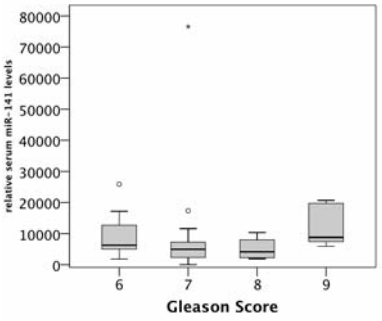

Figure 2. *Serum miR-141 levels were found increased in patients with a high Gleason Score.*

Association of serum miRNA levels with clinicopathological parameters. Finally, we correlated serum microRNA levels in PCA patients with PSA %fPSA levels, clinical tumor stage and Gleason Score respectively. miR-141 Levels were significantly increased in patients with a higher Gleason Score (Figure 2) (p=0.049), but not associated with clinical tumor stage (p=0.148), PSA (p=0.909) nor %fPSA levels (p=0.998). Serum miR-26a-1 levels were neither associated with PSA (p=0.128), %fPSA (p=0.857), tumor stage (p=0.184) nor Gleason Score (p=0.391).

Discussion

The widespread use of PSA testing led to an increase of PCA incidence during the last 30 years. PSA elevation necessitates

prostate biopsy to diagnose PCA. However, prostate biopsy has several drawbacks (false-negative biopsies, under- and over-diagnosis), therefore improved biomarkers are warranted to optimize the clinical management of these patients.

Former studies highlighted various microRNAs in serum/plasma as promising biomarkers for prognosis and diagnosis of PCA (4-6, 9-11, 28). The primary end-point of the present study was to evaluate the diagnostic information of miR-26a-1 (22) and miR-141 (9, 15, 25), earlier shown to be increased in the circulation of patients with PCA. Among 133 patients with evaluable serum microRNAs, we neither saw a diagnostic role for miR-26a-1 nor for miR-141, as its levels were similar in patients with and without PCA in the biopsy. This is contradictory to former studies, but several important differences may explain this finding: earlier studies suffered from smaller sample sizes (*i.e.* 15 to 70 patients (9, 15, 25, 28). Furthermore, PCA patients were compared to healthy subjects (9, 11, 15, 25) or patients with benign prostate hyperplasia (6, 22, 28), and these inadequate controls could lead to an overestimation of the discriminative power of circulating microRNAs. It should also be noted that the inclusion of a relatively large number of locally-advanced or even metastatic (9, 15) PCA may also cause bias; *e.g.* circulating miR-141 levels were increased in patients with aggressive PCA (9, 15, 24, 28). In addition, various technical aspects, *i.e.* isolation procedures (9, 15, 23-25, 28), employment of a pre-amplification reaction (9, 24-26, 28), different PCR chemistry: TaqMan probe (9, 24, 26, 28) *vs.* SYBR Green dye (22, 25)) and reference genes (cel-miR-39 (9, 22, 24, 26, 28) *vs.* RNU1A (25)) could also be cansal of different results. Thus, standardized experimental procedures, and well-defined and clinically-relevant study cohorts are urgently needed for future studies investigating the diagnostic potential of circulating serum/plasma microRNAs.

The second aim of our study was to investigate whether circulating microRNAs could be useful to predict aggressive PCA; earlier reports indicated that circulating miR-26a-1 and miR-141 were associated to stage (25, 28), grade (23, 24) and cancer recurrence (26). In our cohort, miR-141 levels were correlated with the Gleason Score, thereby supporting the prognostic relevance of this microRNA. The analysis of circulating microRNAs could therefore be helpful to identify patients, who benefit from radiation or prostatectomy.

Certain limitations of our study should be mentioned: the PSA and %fPSA levels did not differ between patients with positive and negative biopsies. However, PSA elevation was the most important reason for scheduling prostate biopsy, and this is most probably the reason responsible for the "diagnostic failure" of PSA in our study. We were also surprised of the variable RNA isolation efficiency; earlier microRNA studies performed at our laboratory (22, 27, 29-32) had considerably lower variations. RNA isolation efficiency was not related to the origin of the sample, the storage period or prostate dignity. In order to minimize the potential bias due to variable RNA isolation efficiency, we used a relative quantification using the reference genes *RNU1-4* and *SNORD34*, which were earlier shown to be stably-expressed in serum of patients with urological malignancies (27).

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