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Plasma microRNA signature is associated with risk stratification in prostate cancer patients

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The aim of this study was to establish a unique expression profile of circulating cell-free microRNAs (miRNAs) capable of differentiating between prostate cancer (PCa) patients with high-risk and intermediate-risk Gleason scores. MiRNA expression profiles were determined in plasma samples from 79 treatment-naïve PCa patients, 1–2 follow-up samples after radical prostatectomy (RP) from 51 out of the 79 PCa patients, and 33 healthy men, using a quantitative real-time PCR-based array containing 48 selected miRNAs. We identified 27 up- and 2 downregulated plasma miRNAs in PCa patients compared with healthy men. Most of the upregulated miRNA levels were also associated with increasing PSA levels and Gleason scores. Particularly, the levels of miR-16 ($p = 0.002$), miR-148a ($p = 0.006$) and miR-195 ($p = 0.006$) significantly correlated with high-risk Gleason scores, whereby miR-148a ($p = 0.003$) was also significantly associated with increasing PSA values. The high miRNA levels before RP remained increased in the postsurgical plasma samples. Our findings show a network of deregulated plasma miRNAs. In particular, miR-16, miR-148a and miR-195 are involved in the regulation of the PI3K/Akt signaling pathway. These miRNAs may be promising therapeutic targets for high-risk PCa stratification.

In industrialized countries, prostate cancer (PCa) is the most common malignancy in men. Current diagnostic tests for PCa have low specificity and poor sensitivity. The measurement of prostate-specific antigen (PSA) is still the current gold standard for diagnosis and response to treatment. However, the PSA test has limitations because elevated serum PSA values are not only specific to PCa, and may also be raised in benign prostate diseases due to their association with prostate volume.[1] Although since its introduction, the incidence of PCa nearly doubled, the mortality decreased only modestly. Unfavorably, PSA screening may lead to the detection of clinically insignificant PCa and the problematics of overdiagnosis and radical prostatectomy (RP).[2] Apart from the widespread PSA screening, the Gleason score system is commonly used to predict disease risk and select the therapy for PCa patients. The Gleason system groups PCa patients into low-, intermediate- and high-risk cases. Low/intermediate- and high-risk diseases are most encompassed by men with Gleason of 6–7 and 8–10, respectively. High-risk PCa patients are at higher risk for biochemical recurrence, metastasis and death after RP. However, biopsy Gleason scores may have a significant rate of upgrading.[3]

Based on their biological functions and the possibility to quantify them in real time in patient blood, circulating microRNAs (miRNAs) could become promising candidate biomarkers for PCa,[4] to extend PSA screening and Gleason scoring. These highly stable circulating small noncoding RNA molecules frequently display deregulated expression profiles in the blood of cancer patients, as they can be located in fragile chromosomal regions harboring DNA amplifications, deletions or translocations.[5] As evolutionary conserved family, miRNAs inhibits post-transcriptionally expression of their target genes. Their binding to complementary sequences in the 3′ untranslated-region (3′UTR) of their target mRNAs results in suppression of protein expression or cleavage of their mRNAs.[6] Identification of their oncogenic and tumor suppressive behavior has revealed that many miRNAs are strictly expressed in tumor type- and stage-specific manner contributing to tumor progression and metastasis.[4]

Key words: circulating cell-free microRNAs, radical prostatectomy, Gleason scores, high-risk patients, intermediate-risk patients, PI3K/Akt signaling pathway
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What's new?

Existing diagnostic tests for prostate cancer are suboptimal in specificity and sensitivity, raising the risk of overdiagnosis and overtreatment. To circumvent these issues, novel tumor- and stage-specific biomarkers that can be incorporated into diagnostic testing are needed. This study describes a unique expression profile of circulating microRNAs (miRNAs) in prostate cancer patients that effectively differentiates between patients at intermediate versus high risk for recurrence or death after surgery. In particular, miR-16, miR-148a, and miR-195 were strongly associated with high-risk Gleason scores. The data suggest that specific plasma miRNAs can help identify high-risk patients and potentially facilitate treatment decisions.

In this study, we quantified 48 circulating plasma miRNAs, exclusively selected for PCa, and evaluated them for their diagnostic significance in PCa patients.

Patients and Methods

Study populations

One hundred and forty-four plasma samples of 79 primary PCa patients were collected from January 2013 to May 2015, following patient consensus according to IRB (Institutional Review Board) protocols approved by the University of Manitoba at Winnipeg, Canada. These patients obtained either RP or were followed by active surveillance. The blood samples were collected directly before RP or as part of their active surveillance. The first and second follow-up samples from the PCa patients were collected 2 and 5–6 months after RP, respectively. The median follow-up time was 10 months (range 1–19 months). Median age of PCa patients was 64 years, and ranged from 50 to 90 years. Detailed patient characteristics are summarized in Table 1. In addition, plasma samples were collected during 2016 from 33 healthy men (median age 59, range 48–69) with no history of cancer and in good health based on self-report. Regarding blood processing, uniform management concerning the specific described protocols was performed.

Preparation of plasma and verification of hemolysis

Three milliliters of whole blood as a source of circulating, cell-free nucleic acids were collected in BD Vacutainer tubes (Becton, Dickson and Company, Franklin Lakes, USA) containing ethylendiaminetetraacetic acid (EDTA) and centrifuged at 2000 rpm, 4°C, for 10 min. The upper phase contained blood plasma which was further centrifuged at 3500 rpm for 15 min.

To avoid quantifying miRNAs in hemolytic plasma samples that may influence our results, we performed hemoglobin measurements by spectral analysis.[7] In 7 ml of whole blood, red blood cells were lysed by erythrocyte lysis buffer (containing 0.3 M sucrose, 10 mM Tris pH 7.5, 5 mM $MgCl_2$ and 1% Triton X100). A dilution series (1:1, 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14, 1:18, 1:20, 1:22) of lysed red blood cells in plasma was prepared that served as a standard curve for the measurement of hemolysis of plasma samples from PCa patients (including some follow-up samples). For the measurement of hemolysis, 50 µl of each plasma sample (standard and plasma of interest) were measured in duplicates on a Microplate reader (Tecan, Männerdorf, Switzerland). Absorbance peaks at 414, 541 and 576 nm were indicative for free hemoglobin, with the highest peak at 414 nm. The higher the absorbance in samples is the higher is the degree of hemolysis. The average values and standard deviations were calculated from the duplicates (Supporting Information, Fig. S1).

Isolation of miRNAs by the ABC purification kit

To optimize miRNA quantification with the array cards, we evaluated several RNA extraction kits from different companies (Qiagen, Macherey Nagel, Analytik Jena, ThermoFisher Scientific). We got the highest miRNA amounts from plasma by using the TaqMan miRNA ABC Purification Kit (ThermoFisher Scientific, Darmstadt, Germany) and extracting plasma RNA according to the manufacturer's recommendations. Using the TaqMan miRNA ABC Purification kit (ThermoFisher Scientific) for all plasma samples, cell-free miRNAs were isolated from 50 µl of plasma samples by magnetic beads. Briefly, plasma samples were lysed by adding 100 µl ABC buffer. MiRNAs were isolated by hybridization the lysis to superparamagnetic Dynabeads which are bound to a set of 377 anti-miRNA oligonucleotides. As a positive control for isolation efficiency, the samples were spiked with 2 µl cel-miR-39–3p prior to isolation. After hybridization, the beads were washed and the miRNAs were eluted in 20 µl elution buffer.

cDNA conversion

The extracted miRNAs were immediately reverse transcribed into cDNA using a modified protocol of TaqMan MicroRNA Reverse Transcription kit (ThermoFisher Scientific). The 15 µl reaction containing 6 µl Custom RT primer pool, 0.3 µl dNTPs with dTTP (100 mM), 3 µl MultiScribe Reverse Transcriptase, 1.5 µl 10× RT buffer, 0.2 µl U RNase Inhibitor and 4 µl miRNAs was carried out at 16°C for 30 min, 42°C for 30 min and 85°C for 5 min on an MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation, Ramsey, Minnesota, USA). To avoid false-positive data (e.g., primer dimer formation or unspecific PCR products), a negative control without any templates was included from the starting point of reverse transcription.

Preamplification of miRNAs

To avoid an underpresentation of some miRNAs, a preamplification step of cDNA was included using the TaqMan PreAmp Master Mix Kit. This kit was especially validated for miRNA quantification using Custom TaqMan microRNA

Table 1. Characteristics of prostate cancer patients

Parameters	Patients (%)
Prostate cancer patients	79 (100)
• Patients without follow-up samples	38 (48)
• Patients with one follow-up sample	17 (22)
• Patients with two follow-up samples	24 (30)
Age	64 years (range of 50–90 years)
PSA, U/ml	
<10	50 (63)
>10	29 (37)
Grading	
Low-intermediate grade	32 (41)
High grade	47 (60)
Gleason score	
7, intermediate risk	54 (68)
8,9, high risk	25 (32)
Tumor stage	
pT1	35 (44)
pT2	39 (49)
Unknown	5 (6)
Family history	
No	19 (24)
Yes	49 (62)
Unknown	11 (14)
Smoking	
Smokers	6 (8)
Nonsmokers	46 (58)
Exsmokers	23 (29)
Unknown	4 (5)
Consumption of alcohol	
Daily	7 (9)
Weekly	17 (22)
Occasionally	21 (27)
None	26 (33)
Unknown	8 (10)

Array Cards (ThermoFisher Scientific), and worked together with the TaqMan Gene Expression Master Mix, to create an optimal workflow for small sample amounts. It equally pre-amplifies cDNA targets without distortions in a linear dynamic range. Five microliters of cDNA was preamplified in a 25-µl reaction containing 12.5 µl TaqMan PreAmp Master Mix and 3.75 µL Custom PreAmp Primer Pool (Thermo-Fisher Scientific). PCR was run on an MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation): 1 cycle at 95°C for 10 min, 55°C for 2 min and 72°C for 2 min; 16 cycles at 95°C for 15 sec and 60°C for 4 min; a terminal cycle at 99.9°C for 10 min.

miRNA expression profiling

miRNA profiling was done using the Custom TaqMan microRNA Array Cards (ThermoFisher Scientific), a quantitative real-time PCR-based array containing assays for the detection of 42 human miRNAs of interest, 4 endogenous reference miRNAs (RNU6, RNU44, RNU48, miR-484) and 1 exogenous reference miRNA (cel-miR-39) for data normalization, and 1 assay with an N/A-4343438-Blank (negative control). The selection of miRNAs was performed by reviewing data in PubMed. We selected 42 miRNAs which have been described to be clinically relevant and with an exclusive consideration for PCa. These miRNAs of interest were then mounted on the array cards by the company (ThermoFisher Scientific) and are as follows: let-7c, let-7f-5p, miR-15a-5p, miR-15b-5p, miR-16, miR-20a-5p, miR-21–5p, miR-25–3p, miR-26a-5p, miR-26b-5p, miR-30c-5p, miR-32–5p, mmu-miR-96–5p, miR-100–5p, miR-106b-5p, miR-130b-3p, miR-135b-5p, miR-141–3p, miR-148a-3p, miR-181a-5p, miR-182–5p, miR-183–5p, miR-184, miR-194–5p, miR-195–5p, miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-202–3p, miR-210, miR-218–5p, miR-221–3p, miR-224–5p, miR-331–3p, miR-361–5p, miR-375, miR-425–5p, miR-429, miR-455–5p, miR-494, miR-616–5p and miR-888–5p.

For miRNA array analyses, we modified the protocol of ThermoFisher Scientific. The 112.5 µl PCR reaction contained 56.25 µl TaqMan Universal Master Mix II and 2 µl PreAmp product, and was loaded on the array cards. PCR was run on a 7300 HT 384 block (Applied Biosystems): 1 cycle at 95°C for 10 min; 40 cycles at 95°C for 15 sec and 60°C for 1 min.

Data normalization and statistical analyses

The statistical analyses were performed using the Thermo Fisher Scientific Analysis Software, Relative Quantification Analysis Module, Version 3.1 (www.aps.thermofisher.com), and SPSS software package, version 22.0 (SPSS Inc. Chicago, IL).

First, the obtained data of the miRNA expression levels were calculated and evaluated by the ΔCq method as follows: ΔCq = mean value Cq (miR-484 and cel-miR-39) − mean value Cq (miRNA of interest). As, surprisingly, RNU6, RNU44 and RNU48 were not detectable in our plasma samples, miR-484 and cel-miR-39–3p were used as references for data normalization. To verify whether normalization of the expression data of miRNAs of interest was accurate, we analyzed the miR-484 and cel-miR-39 expression levels, and found that the levels remained relatively constant across the plasma samples.

The Thermo Fisher Scientific Analysis Software was used for performing hierarchical clustering (heat map) and volcano plots. Distances between samples and assays were calculated for hierarchical clustering based on the ΔCq values using Pearson's Correlation. Clustering method was average linkage. Subsequently, the relative expression data were

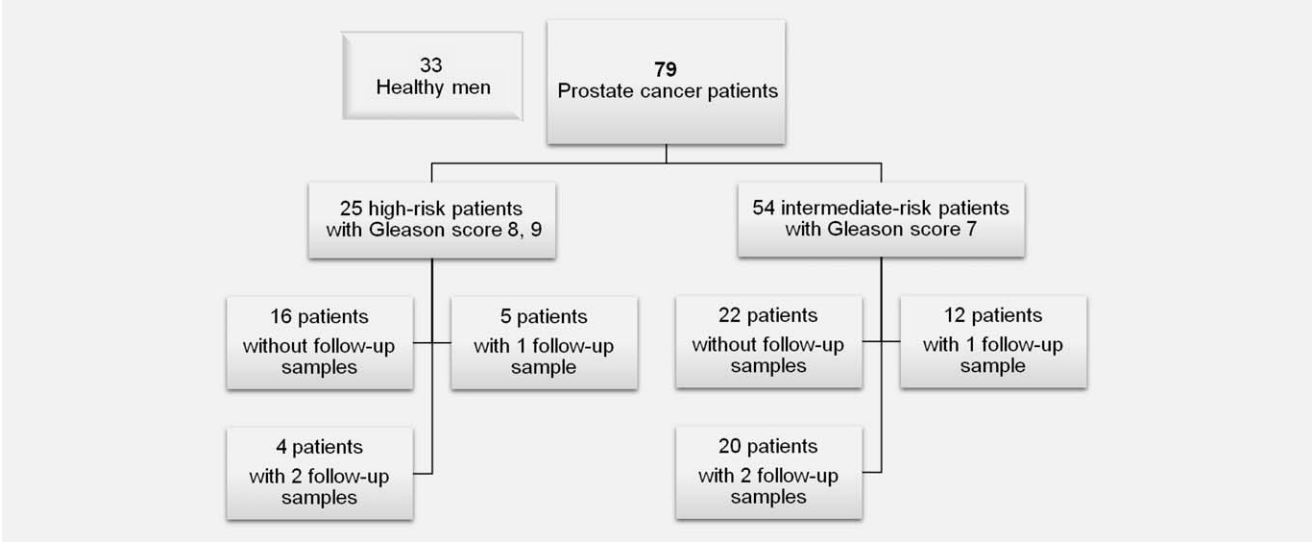

Figure 1. Classification of PCa patients.

log2(ΔCq) transformed to obtain normal distribution data. The confidence of log2(ΔCq) data were verified by amplification curves and Cq confidence (0–1, whereby 1 refers to the highest confidence). Our data showed a Cq confidence of 0.95. Values below 0.95 were discarded. Statistical difference of miRNA expressions between healthy controls and PCa patients was calculated using two-tailed Student's *t* test and depicted as a volcano plot. Our further analyses were performed by the SPSS software. Because of the skewed distribution of the miRNAs concentrations, differences in group levels for nonparametric comparisons were bivariately assessed by univariate analyses of the Mann–Whitney *U* test of two independent variables and Wilcoxon test of two dependent variables. Missing data were handled by pairwise deletion. A p value of <0.05 was considered as statistically significant. All p values are two-sided. Finally, we used the software KEGG-mirPath v.3 to identify relevant mRNA targets and biological pathways.[8]

Results

Patients

We quantified circulating cell-free miRNAs in plasma of 79 PCa patients with follow-up samples and 33 healthy men using quantitative real-time PCR-based array cards, mounted with 42 miRNAs of interest and 6 references which we exclusively selected for our analyses. The candidate miRNAs represent cancer-specific functions *in vitro* or *in vivo*.[4] As shown in the diagram of Figure 1, 25 and 54 of the 79 PCa patients, who were before RP, were high-risk and intermediate cases, respectively. One or two follow-up plasma samples after RP were available from more than half of the patients (Fig. 1). Table 1 summarizes the clinical characteristics and established risk factors of the PCa patients.

Plasma miRNA profiling

Deregulated miRNAs were detected by the highest absolute value of logarithmized fold changes in comparison of PCa patients with healthy men. The estimated raw p values were adjusted for multiple testing, to control the false discovery rate. Table 2 shows the differentially expressed miRNAs with the adjusted p values and the fold changes of plasma miRNAs levels. Whereas 27 miRNAs were upregulated, only 2 miRNAs were downregulated (Table 2). The reason for the majority of upregulated miRNAs is due to our selection of miRNAs which are upregulated in cancer.[4]

A similarity matrix was generated containing all pairwise similarities of the plasma samples of PCa patients and healthy controls. To detect potential clusters in rows (miRNAs) and columns (plasma samples) of the normalized expression matrix, hierarchical clustering was carried out (heat map, Fig. 2).

In the volcano plot of Figure 3, the log2 fold changes are plotted on the x axis and the negative log10 p values are plotted on the y axis. In comparison with healthy men, all down- and upregulated plasma levels of miRNAs in PCa patients are on the left and right side, respectively. Most upregulated plasma miRNAs were miR-221 (17.6-fold), miR-425 (10.7-fold), miR-494 (10.5-fold), miR-361 (9.7-fold) and miR-130b (9.1-fold) (Table 2). These findings indicate that plasma levels of these miRNAs are tremendously increased in PCa patients ($p = 0.0001$).

Steady increase of plasma miRNA levels after RP

To determine the impact of RP on the plasma miRNA levels, we compared the relative miRNA concentrations of the plasma samples prior to RP with those of the follow-up plasma samples after RP. The first follow-up sample was usually collected 2 months after RP and the second follow-up sample 5–6 months after RP. As shown in Table 2, the

Table 2. Significant correlations of plasma levels of circulating miRNAs between PCa patients and healthy men and clinicopathological parameters of PCa patients

			Upregulated miRNAs																									Downregulated miRNAs	
Comparison of miRNA levels		Number of patients		let-7c	let 7f	miR-15a	miR-15b	miR-16	miR-20a	miR-21	miR-25	miR-26a	miR-26b	miR-30c	miR-100	miR-106b	miR-130b	miR-148a	miR-181a	miR-194	miR-195	miR-200b	miR-200c	miR-210	miR-221	miR-224	miR-331	miR-361	miR-425	miR-494	miR-375	miR-183
Healthy vs patients		33 v s 79	fold	3.3	2.3	7.3	6.7	2.0	5.6	7.3	2.1	3.5	3.0	3.0	1.9	5.1	9.1	4.4	6.0	1.6	2.1	4.6	4.6	2.7	17.6	3.3	4.1	9.7	10.7	10.5	0.2	0.4
			p-value	**0.0001**	**0.01**	**0.008**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	0.05	**0.0001**	**0.0001**	**0.0001**	**0.0001**	0.180	**0.0001**	**0.001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.012**
Healthy vs 1st postoperative samples after RP		33 vs 17	fold	3.6	2.8	9.3	6.9	./.	5.9	8.6	2.9	4.8	4.6	4.9	./.	6.9	9.2	5.3	5.0	./.	./.	5.7	6.0	2.8	20.2	5.0	6.0	10.7	7.7	9.5	0.2	./.
			p-value	**0.0001**	**0.005**	**0.011**	**0.0001**	./.	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	./.	**0.0001**	**0.0001**	**0.0001**	**0.0001**	./.	./.	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.028**	**0.0001**	**0.0001**	./.
Healthy vs 2nd postoperative samples after RP		33 vs 24	fold	4.1	5.0	15.2	9.2	1.6	4.7	6.2	2.6	7.6	6.8	7.4	2.9	7.0	6.6	4.1	6.1	3.3	./.	6.4	8.6	2.7	16.5	4.4	9.7	9.9	./.	9.9	0.3	./.
			p-value	**0.0001**	**0.0001**	**0.001**	**0.0001**	**0.023**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.016**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.001**	./.	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	./.	**0.0001**	**0.034**	./.
Clinical parameters																																
PSA	<10	50		0.062	0.198	0.222	**0.002**	**0.037**	**0.024**	**0.040**	**0.009**	**0.007**	**0.024**	**0.010**	**0.016**	**0.002**	0.210	**0.003**	0.551	0.057	**0.021**	**0.030**	**0.017**	0.191	0.062	**0.047**	**0.020**	0.102	0.662	0.085	0.653	0.158
	>10	29																														
Grading	Low-intermediate	32		0.155	0.101	0.714	0.366	0.123	0.144	0.112	0.129	0.730	0.118	0.171	0.845	0.154	0.103	0.664	0.135	0.345	0.197	0.230	0.312	0.366	0.158	0.365	0.329	0.149	0.927	0.298	0.914	0.727
	High risk	47																														
Gleason score	7	54		0.065	**0.043**	0.111	**0.020**	**0.002**	**0.013**	**0.049**	0.076	**0.019**	**0.037**	**0.029**	0.401	**0.010**	**0.038**	**0.006**	**0.019**	0.115	**0.006**	0.050	0.151	0.086	0.091	0.068	0.172	0.094	0.412	**0.026**	0.622	0.116
	8.9	25																														
Tumor stage	pT1	35		0.208	0.091	0.548	0.189	0.502	0.401	**0.038**	0.254	0.274	0.185	0.158	0.061	0.389	0.210	0.109	0.507	0.226	0.596	0.994	0.460	0.401	0.294	0.962	0.326	0.371	1.000	0.695	0.883	0.992
	pT2	39																														

Significant *p* values in bold.

Figure 2. Hierarchical cluster of plasma miRNAs. The heat map of miRNA arrays was performed using quantitative real-time PCR-based array cards mounted with assays for detection of 48 different miRNAs and using 177 plasma samples from 79 PCa patients and 33 healthy men. The colored representation of samples and probes is ordered by their similarity. The red and green colors indicate that the ΔCq value is below (relatively high expression) and above (relatively low expression levels) the median of all ΔCT values in the study, respectively. On top: clustering of samples. On the right side: clustering of probes. The scale bar provides information on the degree of regulation. [Color figure can be viewed at wileyonlinelibrary.com]

plasma levels of the upregulated miRNAs in the presurgical patients usually remained increased in the first and second postsurgical samples after RP. Whereas the plasma levels of the downregulated miR-375 remained downregulated in the postsurgical samples, the downregulated levels of miR-183 raised to normal plasma levels as observed in healthy men (Table 2).

Associations of plasma miRNA levels with the established risk factors

Table 2 summarizes the significant correlations (in bold) between the plasma miRNA levels and the clinicopathological parameters of PCa patients. PCa patients were subgrouped according to low-risk and high-risk factors: PSA values of <10 ng/ml and >10 ng/ml; Gleason scores of 7 (intermediate risk) and 8, 9 (high risk); tumor stages of pT1 and pT2, respectively. The plasma levels of 16 and 15 miRNAs of the 27 upregulated miRNAs significantly correlated with patients' PSA values and Gleason scores, respectively. In particular, the levels of miR-15b ($p = 0.002$), miR-106b ($p = 0.002$) and miR-148a ($p = 0.003$) were most significantly associated with higher PSA values, while the levels of miR-16 ($p = 0.002$), miR-148a ($p = 0.006$) and miR-195 ($p = 0.006$) correlated highly significantly with high-risk Gleason scores. From this, we deduce that the plasma levels of miR-148a were most significantly associated with both clinical parameters. The reason that only one miRNA (miR-21, $p = 0.038$) correlated with tumor stages could be that our cohort contained only PCa patients with early tumor stages pT1 and pT2. No miRNAs were associated with grading, family history, smoker status and alcohol status (Tables 1 and 2).

miRNA targets and signaling pathways associated with high-risk PCa patients

As the Gleason grading system is an important determinant of treatment decisions and prognosis in PCa,[3] we decided to examine which signal transduction pathways miR-16 ($p = 0.002$), miR-148a ($p = 0.006$) and miR-195 ($p = 0.006$), associated most significantly with high-risk Gleason scores, are involved in, and which targets can possibly be regulated by these miRNAs. Using KEGG-mirPath software, we found that these miRNAs participate mainly in the phosphatidylinositol 3-kinase/AKT (PI3K/AKT) signaling pathway which is associated with the progression of PCa.[9]

Discussion

In this study, we identified 27 upregulated and 2 downregulated circulating miRNAs in the plasma samples from PCa patients compared to those from healthy men. Most of these miRNAs were previously reported to be associated with cancer, and several of them have been shown to play a role in PCa.[4,10] In particular, the plasma levels of miR-221, miR-425, miR-494, miR-361 and miR-130b showed the clearest discrimination between healthy men and PCa patients. Moreover, the plasma levels of miR-148a were strongly associated with increasing PSA values and high-risk Gleason scores.

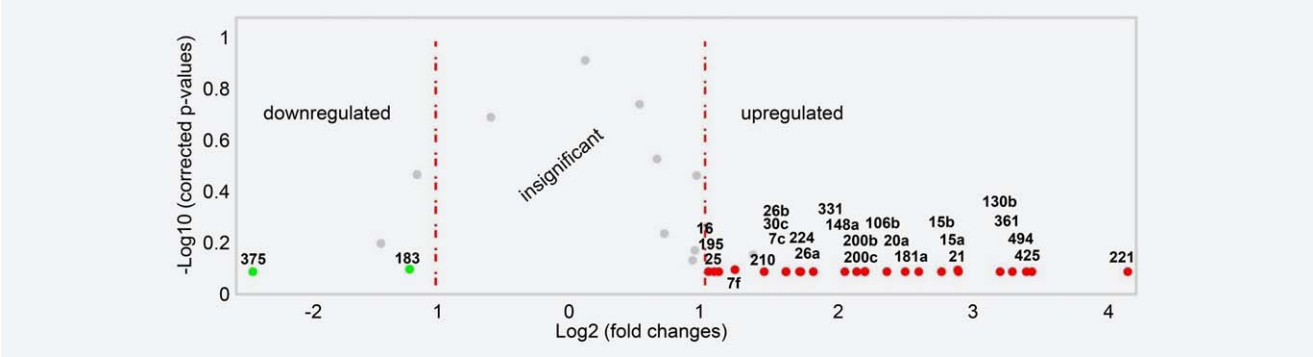

Figure 3. Volcano plot of plasma miRNAs. The plot was drawn for comparison of miRNAs between healthy men and PCa patients. On the left side: downregulated plasma miRNAs. On the right side: upregulated miRNAs. A volcano plot is constructed by plotting negative Log10 of the corrected *p* values on the *y* axis. This results in data points with low *p* values (highly significant) appearing horizontally toward the right and left side of the plot for up- and dowregulated miRNAs, respectively. The *x* axis is Log2 of the fold changes between the two conditions. Each red, green, and grey point refers to an upregulated, downregulated, and insignificant miRNA value, respectively. The grey points are higher in terms of the fold changes (than the other points), but their *p* values are not significant. [Color figure can be viewed at wileyonlinelibrary.com]

Statistical analyses identified this miRNA to be involved in the PI3K/Akt signaling pathway that is frequently deregulated in PCa.

In our study, we detected the clearest discrimination between intermediate- and high-risk Gleason scores and PSA values by the plasma levels of miR-148a, suggesting its crucial role in PCa. The significant upregulation of miR-148a in PCa patients detected in our analyses is in line with other studies.[11,12] Previously, its correlation with biochemical recurrence of PCa was demonstrated, and importantly was independent of PSA values.[13] The reported association of miR-148a with biochemical recurrence substantiates our finding showing its involvement in high-risk PCa patients who are commonly at higher risk for developing biochemical recurrence. Besides, similar to our plasma analyses, circulating miR-148a in the urine of PCa patients was shown to be also associated with PSA values.[12] Thus, plasma miR-148a may be a potential noninvasive tumor marker that may improve the risk stratification of PCa patients.

Apart from miR-148a, we found that the upregulated plasma levels of miR-15b and miR-106b were also significantly associated with higher PSA values. Consistent with our data, Medina-Villaamil *et al.* detected that circulating miR-15b in whole blood of patients with localized PCa could act as biomarker to identify PCa patients with high PSA values.[14] To date, the association of miR-106b expression with PSA values is not known, but this miRNA is known to be frequently overexpressed in PCa patients.[15,16] In addition, we observed that the upregulated plasma levels of miR-16 and miR-195 in PCa patients were significantly associated with high-risk Gleason scores, and also correlated with PSA levels (with lower significance). Previously, miR-16 was described to act as putative tumor suppressor by targeting the oncogene *BCL2* in PCa, and its loss was associated with prostate metastases.[17,18] In high-grade prostate intraepithelial neoplasia (HGPIN), miR-16 was overexpressed compared with benign prostate tissue, and subsequently, at the transition between HGPIN and localized invasive carcinoma, there was a loss of miR-16 expression.[19] However, miR-16 has also been described as oncogene and endogenous control for data normalization.[20–22] In osteoclast differentiation and bone metastasis, the increased miR-16 expression has been reported to be a potential therapeutic target and clinical biomarker of bone metastasis.[23] These differential expression profiles of miR-16 in diverse tumors refer to its complex functions in a temporal and local context. The heterogeneity of the studies could also be explained by preanalytical and analytical factors. Besides, our findings showed the correlation of the plasma levels of miR-195 with Gleason scores that deregulation was also detected in the serum of PCa patients.[24] Despite some discrepant reports, the tremendously strong statistical power of our plasma miRNA panel to associate with high-risk Gleason scores suggests its potential value for PCa risk stratification.

Owing to the importance of the Gleason grading system in monitoring PCa, we performed database searches, and found that all three Gleason scores-associated miRNAs (miR-16, miR-148a and miR-195) are involved in the PI3K/Akt signaling pathway, and may modulate the expression of several genes, both tumor suppressor genes and oncogenes, in this pathway. The PI3K/Akt pathway is one of the most frequently activated signaling pathways in PCa and in other cancers. Its activation leads to PCa progression, resistance and metastasis and is characteristic for aggressive PCa.[25] Therefore, understanding of the mechanism underlying PI3K/Akt signaling may help to develop effective strategies to target this pathway in PCa. For example, miR-148a detected as a noninvasive, potentially high-risk factor in our study could target the tumor suppressor *PTEN* (phosphatase and tensin homology deleted on chromosome ten) that downregulation leads to activation of the PI3K/Akt/mTOR pathway in PCa.[26] In this regard, Qingjuan *et al.* recently reported

that miR-148a could directly bind to the 3′-UTR of PTEN and reduce the expression of PTEN in lupus nephritis, resulting in accelerated glomerular cell proliferation and tumor progression.[27] However, to introduce miRNAs into the clinical practice, more research is needed to identify their target mRNAs and understand their functions, as they harbor both oncogenic and tumor suppressive characteristics. The different features of miRNAs suggest that they act on multiple targets in different cellular processes that contribute to PCa development and progression.

In our PCa patient cohort, we found that miR-221, miR-425, miR-494, miR-361 and miR-130b were the most upregulated plasma miRNAs. With the exception of miR-425, miR-494 and miR-361, the deregulation of miR-221 and miR-130b in the plasma of PCa patient has already been described.[28,29] Agaoglu *et al.* demonstrated the upregulation of miR-221 in the plasma of PCa patients.[28] In addition, Sharova *et al.* calculated the ratio of the plasma levels of circulating miR-106a and miR-130b in PCa patients, and found that it predicts the presence of localized PCa.[29]

Furthermore, we also monitored the expression levels of circulating miRNAs in the postsurgical plasma samples collected 2 and 5–6 months after RP, and detected that the postsurgical miRNA levels remained similarly increased to the presurgical levels. Similarly, Egidi *et al.* determined the fluctuations of miR-21 and miR-141 in the serum of PCa patients 1, 5 and 30 days after RP, and showed that the levels of both miRNAs significantly increased at the 5th postoperative day, after which a gradual return to the preoperative levels was recorded.[30] In contrast, our findings show that the miRNA levels were still increased at 5–6 months after RP, and suggest that miRNAs may be involved in postsurgical inflammatory processes caused by RP and therapy.

Conclusion

Our study demonstrates that specific plasma miRNAs are increased in PCa patients compared with healthy men, and appears to distinguish between PCa patients who may require an intervention or active surveillance. In particular, plasma miR-16, miR-148a and miR-195 are significantly associated with high-risk Gleason scores and may have predictive capacity for more aggressive disease in need of RP. Follow-up studies will validate these findings.

Translational Relevance

To our knowledge, this is the first study to demonstrate that plasma miR-16, miR-148a and miR-195 may discriminate between intermediate- and high-risk Gleason scores. Our study highlights the potential utility of this plasma miRNA panel to select PCa patients who might require an intervention or active surveillance. These miRNAs could possibly participate in the PI3K/AKT pathway that activation leads to PCa progression, resistance and metastasis. Therefore, revelation of their function and potential relationship to this signal pathway could help to develop new noninvasive targeted therapies for PCa patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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