

Investigation of miR-21, miR-141, and miR-221 in blood circulation of patients with prostate cancer

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Received: 10 November 2010 / Accepted: 10 January 2011 / Published online: 28 January 2011
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Abstract In addition to their potential as tissue-based markers for cancer classification and prognostication, the study of microRNAs (miRNAs) in blood circulation is also of interest. In the present study, we investigated the amounts of three cancer-related miRNAs, miR-21, -141, and -221 in blood plasma of prostate cancer (PCa) patients. A cohort of 51 patients with PCa was enrolled into the study, and miRNAs were measured in two subgroups, with localized/local advanced or metastatic PCa. A group of 20 healthy individuals served as the control group. miRNAs were quantified from the total RNA fraction using 200 μ l plasma and the small RNA molecule RNU1A as a control for normalizing the miRNA amounts in circulation. We found similar levels of three miRNAs in healthy subjects with median values of 0.039, 0.033 and 0.04, respectively; (p =n.s.). In the patients, the miRNA levels were higher, with miR-21 being the highest (median, 1.51). The miR-221 levels were intermediate (median, 0.71) while the miR-141 displayed the lowest levels (median, 0.051). The differences between the control group and the patients were highly significant for the miR-21 (p <0.001; area under the

curve (AUC), 88%) and -221 (p <0.001; AUC, 83%) but not for the miR-141 (p =0.2). In patients diagnosed with metastatic PCa, levels of all three miRNAs were significantly higher than in patients with localized/local advanced disease where the difference for the miR-141 was most pronounced (p <0.001; AUC, 75.5%). In conclusion, analysis of miR-21, -141, and -221 in blood of PCa patients reveals varying patterns of these molecules in clinical subgroups of PCa.

Keywords Prostate cancer · MicroRNAs · Metastasis · Blood plasma

Introduction

Prostate cancer (PCa) is one of the most prevalent malignant diseases among men in Western countries [1]. The high mortality rate in patients with PCa is associated with metastatic disease which occurs preferentially to bone tissue. Prognostic models based on pre-treatment prostate-specific antigen (PSA) levels, Gleason score, and clinical staging are, in practice, inadequate to accurately predict disease progression [2]. Despite the efficacy of surgery and radiation therapy for treating localized disease and the possibility of earlier diagnosis through testing for serum PSA, up to 30% of treated PCa patients suffer relapse [3]. Therefore, novel predictive markers are needed.

One of the emerging fields in cancer research is microRNAs (miRNAs). miRNAs are small non-protein coding molecules that regulate basic cellular processes including cellular development, apoptosis, and metabolism and are frequently dysregulated in cancer. Several miRNA expression studies and functional experiments in various cancers have shown an important role for miRNAs in cancer initiation and progression and their potential as diagnostic, prognostic, and predictive biomarkers [4].

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Likewise, in prostate cancer, decreased and/or increased expression of many miRNA molecules has been described [5–8]. However, the small number of the studies in the literature is inconsistent as no overlapping expression pattern has been found [9]. The selection of tissue specimens, use of different techniques for miRNA preparation as well as different test platforms are probably the reasons for these discrepancies.

In addition to their potential as tissue-based markers for cancer classification and prognostication, circulating miRNAs in blood of cancer patients deserve particular attention as potential diagnostic and prognostic biomarkers. miRNAs are present in human plasma in a remarkably stable form that is protected from endogenous RNase activity [10] as most of the circulating miRNAs are included in lipid or lipoprotein complexes, such as apoptotic bodies, microvesicles, or exosomes [11]. In 1 mL of serum, sufficient miRNAs are present to analyze miRNA expression patterns [12]. In a recent study including patients with breast, lung, colon, ovarian, and prostate cancer, it has been shown that expression patterns of miRNAs in serum can correctly discriminate between normal and cancer patient samples [12]. In many other studies, individual miRNA genes proved to provide diagnostic and prognostic serum/plasma markers for various cancers [13, 14]. A recent large-scale study investigating 667 different miRNAs in sera of PCA patients has shown that various miRNAs are highly abundant in serum, while miRNA-375 and miRNA-141 are associated with advanced prostate cancer [15].

In the present study, we investigated the pattern of three cancer-associated miRNAs, miR-21, -141, and -221 in the plasma of PCa patients with local/local advanced or metastatic disease.

Materials and methods

A cohort of 51 patients with pathologically confirmed PCA was enrolled into the study. At diagnosis, the median age of the patients was 67. Eighteen patients had localized disease, eight patients had local advanced disease, and 25 patients had metastases to the bone. The median PSA level at diagnosis was 8.2 ng/mL in patients with localized disease, 35.5 ng/mL in local advanced, and 100 ng/mL in metastatic disease. Median Gleason scores in these groups were 6, 8, and 8, respectively. The study was approved by the local ethics committee of the Istanbul Medical Faculty (No. 2009/2562-30).

Following blinded measurements of plasma circulating miRNAs levels, the results were comparatively analyzed in subgroups of patients with local/local advanced ($N=26$) or metastatic PCa ($N=25$). A group of healthy individuals ($N=20$) served as the control group.

Investigation of miRNA abundance in circulating blood

Circulating RNA molecules were extracted from plasma using the Trizol reagent (Applichem, Düren, Germany). Briefly, 200 μ l of plasma samples was mixed with 800 μ l Trizol reagent and homogenized. The mixture was added on 200 μ l chloroform and incubated on ice for 5 min. Following centrifugation at 11,500 rpm for 5 min, the upper RNA phase was transferred into 550 propanol-containing tubes. Following centrifugation, the RNA-containing pellet was washed with 75% ethanol, air dried, and dissolved in polymerase chain reaction (PCR)-grade water.

Complementary DNA (cDNA) was synthesized using the miScript Reverse Transcription Kit (Qiagen, Valencia, CA, USA) according to the instructions of the manufacturer. The kit includes a poly-A polymerase and a reverse transcriptase. The first step adds a poly-A tail to the 3' end of small RNA molecules, while in the second step, the reverse transcriptase converts RNA to cDNA. To quantitate miRNAs, we used miScript Primer Assays (Qiagen) which include a universal primer specific to the poly-A tail and a miRNA-specific primer. SYBR Green (Qiagen) was used as the fluorescent molecule. The amplified PCR product had a size of approximately 80 bp. The small RNA molecule RNU1A (Qiagen) was used as a control.

Real-time PCR was performed using the LightCycler Instrument 1.2 (Roche Diagnostics, Mannheim, Germany). The PCR program included a fast start step of 10 min at 95°C followed by 45 cycles of amplification where each cycle consisted of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 70°C for 10 s.

For quantitation, the comparative Δ Ct method was used. We first generated serial dilutions of a cDNA sample from a healthy control using the primers for the internal control RNU1A and showed that the amplification of dilutions was reduced down linearly (correlation coefficient=0.99). These dilution series were co-amplified in each PCR session, and the expression values of internal control and miRNAs from a given sample were derived from the crossover threshold (Ct) values using this dilution standard. Subsequently, the levels of a given miRNA in circulating blood were normalized using the miRNA/RNU1A ratio. Real-time experiments were performed twice, and the mean values were calculated.

Statistics

The Pearson correlation test was used to evaluate the correlation between the miRNA markers. The Mann-Whitney test was used to assess univariately the differences between the healthy group and PCa patients and between clinical subgroups. Receiver operating characteristic (ROC)

curves were generated to see the power of miRNAs to distinguish the subgroups of PCa patients from each other.

Results

First, we compared plasma levels of the three miRNAs between healthy controls and the patients. In the healthy controls, miR-21, -141, and -221 levels were similar (median relative values, 0.039, 0.033 and 0.04, respectively; p =n.s.; Fig. 1a). In contrast, in the patients, the miRNA levels displayed a strong variation from patient to patient, ranging from 0.01–88 for miR-21, 0–11.5 for miR-141, and 0.01–43 for miR-221. The pattern of the three individual miRNAs among the patients was also different (p <0.05; Fig. 1a). The miR-21 displayed highest levels, with a median value of 1.51; miR-221, intermediate levels (median, 0.71) while in the miR-141, levels were similar to those in the healthy controls (median, 0.051).

The power analysis with a confidence interval of 95% and a statistical power of 50% showed that our sample size

is sufficient to draw statistical conclusions. Thus, the differences in miRNA plasma levels between the control group and the patients were highly significant for the miR-21 (p <0.001) and -221 (p <0.001) but not for the miR-141 (p =0.23). In agreement with this finding, the miR-21 and miR-221 plasma levels were positively correlated (r =0.88) in the whole study group (N =71; Fig. 1b), while between the miR-141 and other two miRNAs, no correlation was observed (Fig. 1c, d). There was no correlation between the miRNA and PSA levels.

Next, we compared miRNA levels in the patients with localized/local advanced PCa with those having metastatic disease. All three miRNAs were present in significantly higher levels in the circulation of the patients with metastasis than in those with local/local advanced PCa (Fig. 2). The median miR-21 value was 1.15 in the patients with localized/local advanced PCa and 2.64 in the metastatic group (p =0.035). miR-141 median values in the same groups were 0.01 and 0.4, respectively, (p <0.001). Circulating miR-221 levels were 0.23 and 1.18, respectively (p =0.01).

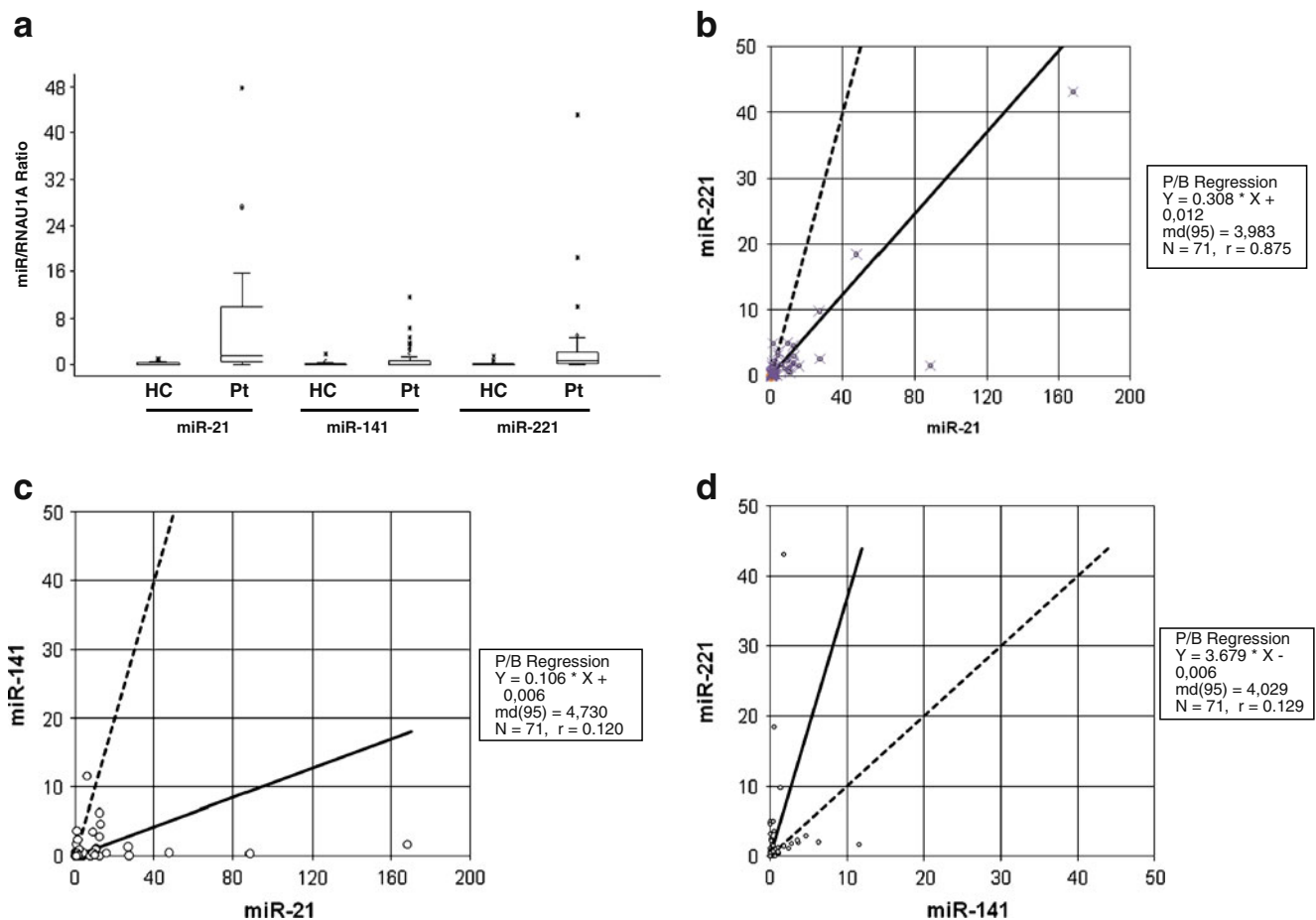


Fig. 1 Amounts of miRNAs and correlations between them. Relative amounts of miRNAs given by the miRNA/RTU1A ratio in healthy subjects and PCa patients (a), Correlation between miR-21 and miR-

221 (b), miR-21 and miR-141 (c), and miR-141, and miR-221 (d). HC healthy controls, Pt patients

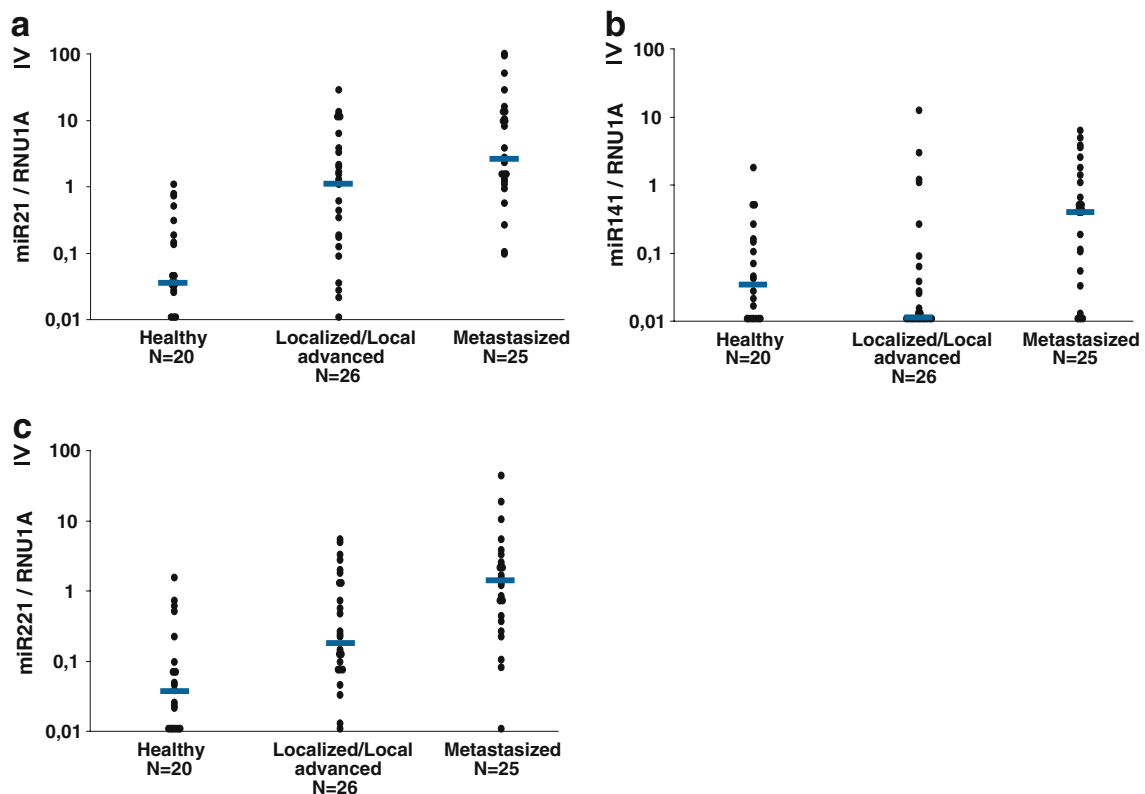


Fig. 2 Amounts of miRNA in subgroups of patients. miR-21 (a), miR-141 (b), and miR-221 (c). Y-axis is scaled logarithmically

Subsequently, we generated ROC curves to see the power of miRNAs to distinguish the subgroups from each other. The miR-21 had the highest area under the curve (AUC; 88%) when all PCa patients were compared to healthy subjects (Fig. 3a). However, when we compared two subgroups of PCa patients, the miR-141 was a better discriminator to distinguish patients with metastases from those with localized/local advanced disease (Fig. 3b). On the other hand, none of the miRNAs did reach the power of

PSA (AUC, 93%) to discriminate metastatic PCa from localized disease.

Discussion

In the present study, we investigated the abundance of three cancer-related miRNAs, miR-21, -141, and -221, in blood plasma of PCa patients. This is the first report to evaluate

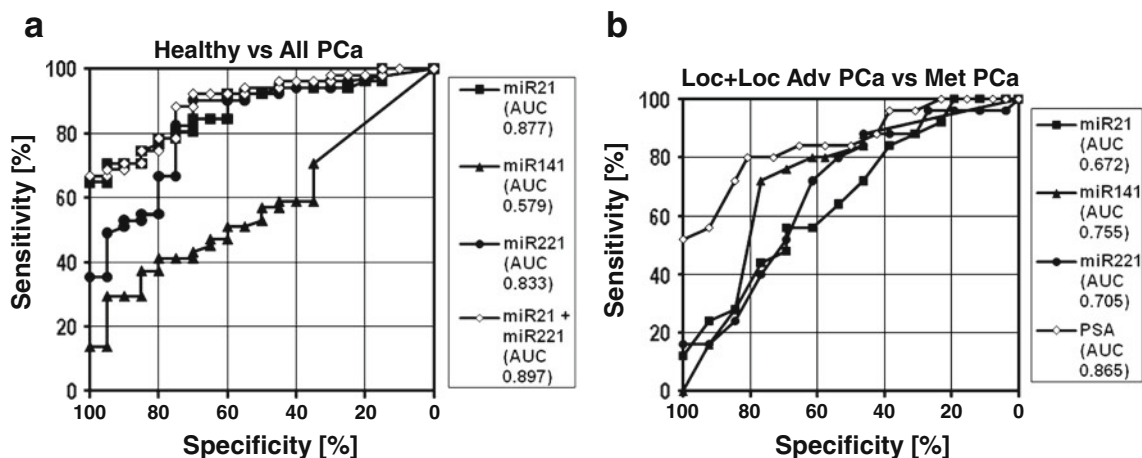


Fig. 3 ROC analysis of miRNAs. Curves for miRNAs to discriminate the whole patients from healthy subjects (a), those with metastatic PCa from localized/local advanced disease (b)

the utility of circulating miRNAs to distinguish PCa patients with metastasis from those with localized/local advanced disease. We found that miR-21 and -221 levels in the patients are higher than in healthy controls, while for the miR-141, no difference was observed. These findings suggest that miR-21 and -221 may be expressed at higher levels in PCa. This is in line with the published data as oncogenic miR-21 has been found to be one of those miRNAs associated with cancer [5]. In early grade prostate tumors, miR-21 levels were found to be elevated when compared to adjacent normal tissue [16]. Upregulation of miR-221 has been described in several types of human tumors, and in patient-derived primary PCa cell lines, high expression has been reported [17, 18]. Our finding that miR-141 levels in the whole patient group are similar to those in healthy controls is in contrast to a previous report which suggested that serum levels of miR-141 can distinguish patients with prostate cancer from healthy controls [10]. However, our sample size is twice as large as compared with this previous report where the difference between the two groups may be mainly attributed to the very broad range in the serum levels. Moreover, the differences between different reports may have been caused by several factors such as use of different analytical protocols or use of serum or plasma for analysis as serum contains higher amounts of circulating nucleic acids [19].

We found that all three miRNAs were present in different levels in the circulation of PCa patients which may indicate a differential expression of these miRNAs in prostate tissue with subsequent release into circulation and/or varying stability in circulation. All three miRNA levels were higher in patients with metastases than in those with localized/local advanced disease. It was conceivable that even if levels of the miR-141 were not different between healthy controls and the patients, among the three miRNAs, it was the most powerful discriminator of metastatic PCa from localized/local advanced disease. This is in agreement with recent reports [12, 15] which found that circulating miR-141 is associated with advanced PCa. Higher abundance of the miR-141 in circulating blood of patients with metastases might also indicate progression of localized disease. This has to be tested in future studies. In some other cancers including colon, breast, lung, and bladder cancer, miR-141 expression in tumor tissues has been shown to be associated with advanced disease [20].

In conclusion, analysis of miR-21, -141, and -221 in blood of PCa patients reveals that miR-21 is more useful to differentiate PCa patients from healthy controls, while the miR-141 was a better discriminator of metastatic PCa from localized/local advanced disease. Our results suggest that analysis of these miRNAs might have clinical utility as a supplement to PSA testing.

Acknowledgements This work was supported by Istanbul University Research Fund (Project no. 7986).

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