Use of MicroRNAs as biomarkers in the early diagnosis of prostate cancer

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Introduction: Prostate cancer (PCa) is a common type of cancer in western countries and prominent cause of mortality in men. The aim of the study was to evaluate miRNAs as biomarkers in PCa in healthy individuals and prostate cancer patients, and examined the effect of miRNA levels on tumour mass. Material and methods: Twenty prostate cases, age (mean and range) 61,4±12.1 (45-73), and twenty healthy men, age 59,3±11.2 (44-70) were included to the study. Seven miRNAs including two internal controls (Let7c, miR125b, miR141, miR145, miR 155, miR181 ve miR192) were evaluated in two groups. Results: The mean and range of prostate specific antigen (PSA) in cancer cases and healthy individuals were 6.79±2.84 ng/ml (2.25-14.7) and 3.8±2.2 ng/ml (1.3-7.8) respectively. The level of miR141 was significantly lower in PCa cases than healthy individuals (p=0,004), and miR155 was significantly higher (p=0,005) in PCa cases. Both miRNAs were explored sensitive and specific in the ROC analysis. Tumour mass were found to be associated with the level of miR-125b and miR-145. Conclusion: validation studies are required in wider patient groups in the subject of tumor effect and miRNA biomarkers in prostate cancer.

Keywords: Prostate cancer, biomarker, microRNA
biomarkers in PCa in healthy individuals and prostate cancer with patients, and examined the effect of miRNA levels on tumour mass. **Materials:** Study Group: The study group consisted of 20 PCa patients and 20 healthy individuals of similar ages. Between 2017 and 2019, blood samples were taken from patients who were newly diagnosed with PCa after prostate biopsy at Akdeniz University Faculty of Medicine (Oncology Department), Antalya Research and Training Hospital (Urology Department) and Antalya Lara Anatolian Hospital (Urology Department). As the control group, similar aged healthy male volunteers who applied to our genetic diagnosis and evaluation center and who had had normal physical examination and laboratory results were selected.

As inclusion criteria, patients were newly diagnosed, and no treatment was started. As exclusion criteria was the patient’s voluntary withdrawal.

**Ethics Committee:** Ethical approval was obtained from Akdeniz University, Faculty of Medicine, Ethics Committee. Each individual was included to the study after he was informed and he signed a written consent form. Then 5 cc of EDTA anti-coagulated blood samples were taken from patients and individuals of control group. An additional 5 cc whole blood samples were also taken from healthy individuals.

**MiRNA selection:** A total of seven miRNAs, let-7c, miR-155, miR-125b, miR-141, miR-145, miR-181 and miR-192 have been selected according to literature review. Mir-181 and miR-192 were endogenous control according to their expression levels.

**Method**

Plasma samples were separated from 2 cc EDTA anti-coagulated blood using a refrigerated centrifugate at 2000xg for 15 minutes. for all patients and healthy individuals. MiRNA isolation from plasma was performed using mirVana™ miRNA Isolation Kit, with phenol (Invitrogen by Thermo Fisher Scientific, Cat. no: AM1560). The concentration of miRNA was measured using Qubit™ microRNA Assay Kit (Invitrogen by Thermo Fisher Scientific, Cat. No: Q32880) and Qubit-3 Fluorometer and noted as ng/µl units. MiRNA samples, that were in the suitable concentration, converted to cDNA using TaqMan™ Advanced miRNA cDNA Synthesis Kit (Invitrogen by Thermo Fisher Scientific, Cat. no: A28007) and Veriti Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific). Two aliquots of cDNA samples were stored at -20°C until the sufficient numbers of samples were collected to study.

TaqMan™ Advanced miRNA assay, TaqMan™ Fast Advanced Master Mix (Applied Biosystems by Thermo Fisher Scientific, Cat. No: A25576 and Cat. No: 4444557 respectively), cDNA and adequate nuclease-free water were mixed to reach a total volume of 20 µl according to the test protocol. Reaction was carried out in duplicates in a 96 well-plate using StepOnePlus™ Real-Time PCR system (Catalog No: 4376598, Thermo Fisher Scientific).

MiRNA expression levels were evaluated using Treshold cycle (C ) values. C values were automatically exported from the system to an excel file. The mean C values of the duplicated samples were calculated and C values were defined by taking into account of the Ct values of endogenous controls miR 181 and miR 192. The C values of the individuals with PCa were compared to those of the healthy control group.

**Statistical Method:** Data were evaluated with SPSS (Statistical Package for the Social Sciences) version 23.0 (SPSS Inc., Chicago, IL, USA) program. Descriptive findings are presented with number, percentage, mean ± standard deviation and median. Shapiro-Wilk test and skewness/kurtosis values were used to evaluate whether the data represented normal distribution. Independent samples-t-test was used if the data conformed to the normal distribution, or Mann-Whitney U test was used when the data were not normally distributed to compare the C values of patient group to those of the control group. To compare C values of the patient group before and after the surgery, Independent dependent samples-t-test was used if the data conformed to the normal distribution, or Wilcoxon signed-rank test was used when the data were not normally distributed. The association of miR-141 and miR-155 with the PSA levels and Gleason scores of patients was evaluated using Spearman Correlation test. The limit value was accepted as p <0.05 for statistical significance.

Receiver study characteristic (ROC): ROC curve analysis was performed to determine the sensitivity and specificity and diagnostic efficacy of miRNAs (7).
Results

The age range of 20 patients included in the study was 45-73 years, and the mean age was 61.4 ± 12.1 years. The age range of the control group was 44-70 years, and the mean age was 59.3 ± 11.2 years. The range of PSA in PCa group was 2.25-14.7 ng/ml and the mean of PSA was 6.79 ± 2.84 ng/ml. The range of PSA in the control group was 1.3-7.8 ng/ml and the mean of PSA level was 3.8±2.2 ng/ml (Table 1).

The Gleason score was 9 in one patient, 7 in twelve patients and 6 in seven patients. In the staging of tumor; two patients were T1b, seven patients were T1c, two patients were T2b, eight patients were T2c and one patient was T3 stage. Lymph node metastasis was detected in one patient, however, there was no distant of disseminated metastasis (Table 2).

MiR-141 delta-Ct value was significantly lower in PCa group compared to the healthy individuals (p=0.004) taking into account the miR-192 as an endogenous control (Table 3).

Table 1. The age and PSA levels of the patient and control groups

| Patient Group (n:20) | Age Range (year) | Age (Mean±SD) | PSA Range (ng/ml) | PSA (Mean±SD) |
|---------------------|-----------------|---------------|------------------|---------------|
|                     | 45-73           | 61.4±12.1     | 2.25-14.7        | 6.79±2.84     |
| Control Group (n:20)| 44-70           | 59.3±11.2     | 1.3-7.8          | 3.8±2.2       |

Table 2. PSA levels, Gleason Scores, Tumor Stages and Metastasis status of PCa cases.

| No | PSA level ng/ml | Gleason Score | Tumor Stage | Lymph Node Metastasis | Disseminated Metastasis |
|----|-----------------|---------------|-------------|-----------------------|-------------------------|
| 1  | 9.4             | 3+4=7         | T1b         | N0                    | M0                      |
| 2  | 6.35            | 3+3=6         | T1c         | N0                    | M0                      |
| 3  | 5.05            | 3+3=7         | T1c         | N0                    | M0                      |
| 4  | 5.39            | 4+3=7         | T2b         | N0                    | M0                      |
| 5  | 6.54            | 3+3=6         | T1c         | N0                    | M0                      |
| 6  | 6               | 3+4=7         | T2b         | N0                    | M0                      |
| 7  | 14.7            | 3+3=6         | T2c         | N0                    | M0                      |
| 8  | 2.25            | 3+3=7         | T1b         | N0                    | M0                      |
| 9  | 4.65            | 3+4=7         | T2c         | N0                    | M0                      |
| 10 | 6.33            | 3+4=7         | T2c         | N0                    | M0                      |
| 11 | 6.4             | 3+3=6         | T1c         | N0                    | M0                      |
| 12 | 11              | 3+4=7         | T2c         | N0                    | M0                      |
| 13 | 5.18            | 3+3=6         | T1c         | N0                    | M0                      |
| 14 | 4.69            | 3+3=6         | T1c         | N0                    | M0                      |
| 15 | 5.1             | 3+4=7         | T2c         | N0                    | M0                      |
| 16 | 6.5             | 3+4=7         | T2c         | N0                    | M0                      |
| 17 | 9.4             | 3+4=7         | T2c         | N0                    | M0                      |
| 18 | 10.4            | 4+5=9         | T3          | N1                    | M0                      |
| 19 | 5.05            | 3+4=7         | T2c         | N0                    | M0                      |
| 20 | 5.39            | 3+3=6         | T1c         | N0                    | M0                      |
| Min Max | 2.25-14.7   | 7 cases: 6   | 2 T1b       | 1 N1                  | 20 M0                  |
|        | 6.79 ±2.84     | 12 cases: 7  | 7 T1c       | 19 N0                 |                         |


MiR-155 delta-Ct value was significantly higher in PCa group compared to the healthy individuals (p=0.005) when taking the miR-181 into account as an endogenous control (Table 4).

MiR-155 delta-Ct value was significantly higher in PCa group compared to the healthy individuals (p=0.005) when taking the miR-181 into account as an endogenous control (Table 4).

The correlation between Gleason scores and miR141 were analysed. When miR-181 was used as an endogenous control (delta181), the results were as follows: r=-0.544, p=0.055. When miR-192 was used as an endogenous control (Delta 192CT), the results were as follows: r=-0.544, p=0.055.

### Table 3. Delta-Ct values of miRNAs in the PCa cases and healthy controls (Delta192CT)

| miRNA | Mean±SD | Median  | p     |
|-------|---------|---------|-------|
| Let7c |         |         |       |
| PCa (n=18) | 3.36518093400±1.671916059308 | 3.84331321700 | 0.799* |
| Control (n=11) | 3.75030201309±4.713550026345 | 2.70037651100 |       |
| Mir125b |         |         |       |
| PCa (n=18) | 5.52881254972±2.014644586382 | 5.11593087500 | 0.257* |
| Control (n=4) | 6.13900000000±4.35251651347 | 6.22800000000 |       |
| Mir141 |         |         |       |
| PCa (n=13) | -1.65941545285±1.742292084281 | -1.79182243300 | 0.004† |
| Control (n=6) | 3.50726966100±2.751098536408 | 4.52700000000 |       |
| Mir145 |         |         |       |
| PCa (n=17) | 3.31963774718±2.743487617645 | 3.18801116900 | 0.194* |
| Control (n=4) | 5.26000000000±1.545657357459 | 5.30000000000 |       |
| Mir155 |         |         |       |
| PCa (n=16) | 7.46429300356±2.990451349413 | 7.46502018000 | 0.563† |
| Control (n=5) | 5.87300005180±4.08885684477 | 7.83100000000 |       |

* Independent samples t test, † Mann-Whitney U test, miR192 was used as an endogenous control (Delta 192CT).

### Table 4. Delta-Ct values of miRNAs in the PCa cases and healthy controls (Delta181CT)

| miRNA | Mean±SD | Median  | p     |
|-------|---------|---------|-------|
| Let7c |         |         |       |
| Prostat ca (n=18) | -1.38913529011±3.369260958702 | -1.29359340700 | 0.329* |
| Control (n=18) | -.36369462594±2.820594259431 | -.19196891800 |       |
| Mir125b |         |         |       |
| Prostat ca (n=18) | .77449632578±2.553251772847 | 1.02641487150 | 0.110* |
| Control (n=5) | -1.25451383060±1.648955025619 | -2.35300000000 |       |
| Mir141 |         |         |       |
| Prostat ca (n=13) | -6.83450448200±4.13259350680 | -7.84710502600 | 0.222* |
| Control (n=11) | -4.55280832573±4.775296604690 | -3.18200000000 |       |
| Mir145 |         |         |       |
| Prostat ca (n=17) | -1.50609656188±2.625061673107 | -1.31096649200 | 0.843* |
| Control (n=4) | -1.80325000000±2.877396551862 | -2.68000000000 |       |
| Mir155 |         |         |       |
| Prostat ca (n=16) | 2.91233301169±2.180038171474 | 2.57591247550 | 0.005* |
| Control (n=7) | -.01914476700±1.764352995848 | -.75000000000 |       |

* Independent samples t test.
as an endogenous control (delta192) the results were as follows: \( r = -0.469, p = 0.106 \).

Correlation analysis between PSA level and miR-155 gave the results of \( r = 0.021 \) and \( p = 0.940 \) for delta181, and \( r = 0.228 \) ve \( p = 0.395 \) for delta 192.

The correlation between Gleason scores and miR155 was assessed. The results were \( r = 0.201, p = 0.456 \) for delta181 miR155, and \( r = 0.460, p = 0.073 \) for delta192 miR155.

Similar to miR141, it was determined that miR-155 values did not correlate with PSA level or Gleason scores.

ROC Analysis: As a result of group comparisons, the diagnostic properties of two miRNAs, which showed a significant difference between PCa cases and healthy controls, were evaluated using ROC analysis. While the closer values of area under the curve (AUC) to 1 indicates the higher diagnostic value of the test, \( p \) value below 0.05 indicates that diagnostic value of the test is statistically significant.

ROC analysis for Delta192CTmiR141 and Delta181CTmiR155 was performed and the AUC, sensitivity, specificity and \( p \) values were given in Table 5. Optimum cut-off value of Delta192CTmiR141 was 2.15087032350, and its sensitivity and specificity were 100.0 and 83.3 respectively. The optimum cut-off value of Delta181CTmiR155 was 0.12874794000, the sensitivity and specificity of Delta181CTmiR155 were 93.8 ve 57.1, respectively.

As a result, two miRNAs, miR-141 and miR-155, may be sensitive and specific biomarkers among the 5 miRNAs (Let7c, miR-125b, miR-141, miR-145 and miR-155) evaluated in prostate cancer patients.

To evaluate the effect of tumor mass on the miRNA levels, DeltaCT values of five miRNAs (let-7c, miR-155, miR-125b, miR-141 miR-145) were analysed before and after the prostate surgery of five PCa patients (Table 6). While miR-181 was used as an endogenous control (Delta181CT), miR-125b and miR145 values were found significantly lower after the prostate surgery (\( p = 0.001 \) and \( p < 0.001 \), respectively). Similarly, when miR-192 was used as an endogenous control (Delta192CT), miR-125-b and miR145 values significantly decreased after prostate surgery (\( p = 0.002 \) and \( p = 0.001 \), respectively) (Table 7).

### Discussion

PCa is common in western countries and is a leading cause of cancer-related mortality in men (1). In a study conducted by Porzycki et al., five miRNAs (miR-106b, miR-141, miR-21, mir-34a ve miR-375) have been analysed in 20 patients, mean age of 68.6 years and PSA level 21.3 ng/ml and eight healthy controls. They found the relative-expression rates of miR-141-3p, miR-21 and miR-375 have been increased 1.9, 2.4 and 2.6 times, respectively in the PCa cases compared to healthy controls. As a result, it was em-

### Table 5. AUCs, sensitivity and specificity of miRNAs in ROC analysis.

| miRNAs        | AUCs | Sensitivity (%) | Specificity (%) | P value |
|---------------|------|-----------------|-----------------|---------|
| Delta192CTmiR141 | 0.923 | 100.0           | 83.3            | 0.008   |
| Delta181CTmiR155 | 0.830 | 93.8            | 57.1            | 0.013   |

### Table 6. MiRNA DeltaCT values (endogenous control miR181) before and after the surgery of 5 patients with PCa

| Before the surgery | Let 7c | mir 125b | mirR 141 | mirR 145 | mirR 155 |
|--------------------|--------|----------|----------|----------|----------|
| Mean               | 2.578701401 | 3.642106247 | -2.61190859450 | 0.924751282 | 4.431347275 |
| Standard Deviation | 1.577204153 | 0.590082027 | 0.753381548125 | 1.49838009 | 2.471392116 |
| Median             | 3.089383028 | 3.515453339 | -2.61190859450 | 1.144018173 | 4.355184555 |

| After the surgery  | Let 7c | mir 125b | mirR 141 | mirR 145 | mirR 155 |
|--------------------|--------|----------|----------|----------|----------|
| Mean               | 1.424925612 | 0.498040771 | -3.664655687 | -2.95640355 | 5.82402153 |
| Standard Deviation | 0.680760615 | 1.124746445 | 0.641376577 | 1.383867105 | 0.97207358 |
| Median             | 1.387573242 | 0.970226288 | -3.664655687 | -2.910028458 | 6.349346161 |

| p                  | 0.080† | 0.001* | 0.180† | <0.001* | 0.198* |

* Dependent samples t test, † Wilcoxon signed-rank test
did not investigate P53 gene mutation or DNA methylation.

In a study conducted by Richardsen et al., using the in situ hybridization method in tumor epithelium and tumor tissues of patients with prostate cancer, it was found that miR141 was higher and miR145 was lower in patients with a Gleason score above 8 and tumor size over 20 mm (11). In present study, miRNAs in plasma samples instead of tumor tissue were analysed using Real Time PCR method, and there was no patient with Gleason score above 8. The lower level of miR141 and normal level of miR145 might be due to the difference in the experimental design.

Guo et al. studied miR-155 gene expression in tumor tissue in a total of 86 PCa patients. They found that the level of MiR-155 was significantly higher in tumour tissue compared to para-carcinoma tissue (p <0.05). PSA and miR-155 expressions were positively correlated with TNM stage and tumour volume (p <0.05). They emphasized that the combination of PSA and miR-155 would help in the early diagnosis of prostate cancer (12). In our study, DeltaCT181 miR-155 levels were higher in the patients.

Filella et al. summarized miRNA expression patterns and their involvement in PCa pathogenesis. Most of the studies have been conducted in serum or plasma but studying miRNAs in the urine sample obtained after prostate massage has been emerging as a new method. Although preliminary results regarding miR-141, miR-375, and miR-21 are promising, larger and prospective studies with standard methodology are required to define the value of miRNAs in the detection and prognosis of PCa (13).

### Table 7. MiRNA DeltaCT values (endogenous control miR192) before and after the surgery of 5 patients with PCa

|                  | Before the surgery | After the surgery |
|------------------|--------------------|-------------------|
|                  | Let 7c             | mir 125b          | miR 141 | miR 145 | miR 155 |
| Mean             | 2,53694152         | 3,600346375       | -1,188178168 | 0,882991409 | 4,389587402 |
| Standart Deviation | 1,304743873       | 0,426558787       | 1,672033822 | 2,085506695 | 1,791578571 |
| Median           | 2,80836105300      | 3,62603950500     | -1,188178168 | 1,201824188 | 4,64254951500 |
| p                | 0,922*             | 0,002*            | 0,180†   | 0,001*   | 0,080†   |

* Dependent samples t test, † Wilcoxon signed-rank test
Liu et al. analysed miR-146a and miR-152 expression in serum of prostate cancer patients and the relationship between their expression and clinicopathological parameters in 56 PCA and 56 healthy volunteers. They used the ROC curve to investigate the diagnostic value of each indicator. They found that miR-146a expression was significantly higher in patients in the cancer group than in the healthy group (p<0.05), and miR-152 significantly lower in the cancer group than in the healthy group (p<0.05). They reported that expression levels of miR-146a and miR-152 were closely related to clinical staging, presence or absence of bone metastasis, tPSA and pathological staging in patients with prostate cancer (p <0.001). They also reported that miR-146a and miR-152 levels were negatively correlated with each other in the serum of patients with prostate cancer. While miR-146a expression level has been upregulated in prostate cancer, miR-152 expression level has been downregulated in prostate cancer (14).

In our study, when the ROC analysis was performed, the sensitivity and specificity of miR-141 and miR-155 were found to be significant. The sensitivity and specificity values for Delta192CT miR-141 were 100.0% and 83%, respectively, and the sensitivity and specificity values for Delta181CTmir155 were 93.8% and 57.1%, respectively.

In a study by Zhu et al., the expression of miR-30c and miR-29b has been evaluated using RT-PCR in cancer tissue and adjacent cancerous tissue after prostatectomy in 187 PCA patients. Their expression has been was reported to be lower in prostate cancer tissue. The association between clinical features and two miRNAs have been evaluated and ROC curve analysis has been was performed. Lymph node metastasis, bone metastasis and Gleason score have been were related to miR-30c and miR-29b expression. As a result, they reported that miR-30c and miR-29b expression might distinguish cancer tissue and paracancerous tissue, and they could also be a new biomarker for the diagnosis of PCA (15). These two miRNAs were not included to our study.

Al-Kafaji et al. conducted a study to evaluate four miRNAs in the differential diagnosis of PCa and Benign Prostat Hyperplasia (BPH). They included thirty-five PCa patients, thirty-five individuals with BPH and thirty healthy individuals in their study. Four miRNAs, miR-15a, miR-126, miR-192 and miR-377, have been downregulated in the PCa patients compared to the BPH and healthy controls. According to tumour stage (T), PSA level, and Gleason score (GS) they have grouped the PCa patients as low risk (T 1/2, PSA<10 ng / ml and GS ≤7) and high risk (T 3/4, PSA>20 ng/ml and GS-8). Four miRNAs, particularly miR-126, have been detected lower in the high risk group compared to low risk group. After ROC curve analysis, they emphasized that miRNAs may provide an important contribution to the differential diagnosis of PCa from BPH, and risk assessment of PCa patients (16). In the our study, nine PCa patients were at T1, ten patients were at T2 and one patient was at T3 stage. Gleason Score was 6 for seven patients, 7 for twelve patients and 9 for one patient. However, miRNA expression levels were not compared according to tumor stage or Gleason score.

McDonald et al. have investigated whether miRNAs could be a potential biomarker in the early diagnosis of PCa in men who underwent prostate needle biopsy. They have included 66 PCa cases and 68 controls (8 men with atypical lesion and 60 men with no PCa). Significant differences have been reported for miR-381, miR-34a, miR-523, miR-365, miR-122, miR-375, miR-125-5b, miR-34b, miR-450b-5p and miR-639 between groups after adjusting for age. However, no miRNAs have been identified for the PCa diagnosis after adjustment for multiple comparisons. They reported moderate positive correlation between serum PSA levels and miR-34a in the patient group, and similar correlation between PSA levels and miR-381 in the patient and control groups (17). In the present study, the expression level of miR-125-5b was not different between case and control groups. However, miR-125-5b deltaCT level was significantly decreased after prostate tumor resection surgery (Table 6 and 7).

Lin et al. evaluated the expression level of miRNAs and mRNAs in primary PCa and metastatic PCa (MPCa) samples using a network vulnerability analysis model. They constructed the MPCa-specific microRNA-mRNA network, and identified five miRNAs (miR-101-3p and miR-145-5p, miR-204-5p, miR-198 and miR-152) as candidate biomarkers for PCa metastasis (18). In the current our study, lymph
node metastasis was detected in one patient. We could not evaluate the association between miRNA level and PCA metastasis.

Aghdam et al. indicated that PSA test was routinely used for the screening and early detection of prostate cancer, however, it was not specific for prostate cancer, resulting in high false-positive rates and poorly correlated with cancer stage. They notified the necessity of a different diagnostic and prognostic marker for PCA (19). The critical role of the miRNAs in the physiological pathways have been explored in various studies. Therefore, irregulation of miRNAs has been related to the development of various diseases including PCA. Various miRNAs (miR-34, miR-21, miR-155, miR-221, miR-222 ve let-7), cellular and molecular pathways (c-Myc, EZH2, c-RSC, BCL2L2, E2F6, ZEB, HMGA251 ve CCND2) play a role in the pathogenesis of PCA. Therefore, miRNA expression profiles could be potential candidates as a biomarker for the diagnosis, treatment and prognosis of the PCA (19).

The most important difference of the current study from previous studies was the evaluation of the effect of tumour mass on miRNA levels. The expression profiles of miRNAs (let-7c, miR-155, miR-125b, miR-141 miR-145) have been evaluated before and after the prostate resection in five PCA patients. Following the surgery, delta CT levels of miR-125b and miR-145 were significantly decreased for both endogenous control miR-181 (Table 6) and endogenous control miR-192 (Table 7).

In conclusion, in the present study, among the five miRNAs, miR-141 and miR-155 were identified as specific and sensitive biomarkers for PCA. In addition, tumour mass may have an effect on miR-125b and miR-145 expression level. Validation studies are required in a larger patient group to reveal the association between biomarkers and tumour effect in PCA.

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