miR-148a, miR-152 and miR-200b promote prostate cancer metastasis by targeting DNMT1 and PTEN expression

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Abstract. MicroRNAs (miRs) modulate the expression of target genes in the signal pathway on transcriptome level. The present study investigated the 'epigenetic-based miRNA (epi-miRNA)-mRNA' regulatory network of miR-34b, miR-34c, miR-148a, miR-152, miR-200a and miR-200b epi-miRNAs and their target genes, DNA methyltransferase (DNMT1, 3a and 3b), phosphate and tensin homolog (PTEN) and NK3 Homeobox 1 (NKX3.1), in prostate cancer (PCa) using reverse transcription-quantitative PCR. The expression level of NKX3.1 were not significantly different between the PCa, Met-PCa and control groups. However, in the PCa and Met-PCa groups, the expression level of DNMT1 was upregulated, while DNMT3a, DNMT3b and PTEN were downregulated. Overexpression of DNMT1 (~5 and ~6-fold increase in the PCa and Met-PCa groups respectively) was accompanied by a decreased expression in PTEN, indicating a potential negative association. Both groups indicated that a high level of DNMT1 is associated with the aggressiveness of cancer, and there is a a directly proportional relationship between this gene and PSA, GS and TNM staging. A significant ~2 to ~5-fold decrease in the expression levels of DNMT3a and DNMT3b was found in both groups. In the PCa group, significant associations were identified between miR-34b and DNMT1/DNMT3b; between miR-34c/miR-148a and all target genes; between miR-152 and DNMT1/DNMT3b and PTEN; and between miR-200a/b and DNMT1. In the Met-PCa group, miR-148a, miR-152 and miR-200b exhibited a significant association with all target genes. A significant negative association was identified between PTEN and DNMT1 in the Met-PCa group. It was also revealed that that miR-148a, miR-152 and miR-200b increased the expression of DNMT1 and suppressed PTEN.

Furthermore, the 'epi-miRNA-mRNA' bidirectional feedback loop was emphasised and the methylation pattern in PCa anti-cancer therapeutics was highlighted.

Introduction

Tumor suppressors, proto-oncogenes and epigenetic factors serve a central role in carcinogenesis. Instead of a treatment that also harms healthy cells, such as chemotherapy, epigenetic therapy has led to the development of a new therapeutic option in which side effects are minimized (1). Prostate cancer (PCa) is a disease with multifactorial etiology, including epigenetic and genomic alterations (2,3). While the traditional indicator in the diagnosis of PCa is the PSA level in the blood, this marker may also increase in other diseases related to the prostate (such as prostatitis and benign prostatic hyperplasia) (4). Moreover, there is no direct association between PSA level and the stage of PCa. For the clinical management of PCa, reliable new diagnostic, predictive, prognostic and therapeutic biomarkers are needed (5-7).

Micro RNAs (miRNAs/miRs) are non-coding (ncRNA) RNAs 20-25 nucleotides in length that function at the post-transcriptional stage as regulators of gene expression (6,8,9). Depending on whether miRNA is complementary with the target mRNA, the latter is degraded or translation is ceased (6). The role of miRNAs in the epigenetic mechanism is among the most interesting subjects of research in cancer studies (10). Despite numerous challenges in their clinical application, miRNAs have the potential to reduce off-target effects and complete traditional, targeted or immune-based therapies for cancer (11). Multiple studies have been conducted to elucidate the association between prostate carcinogenesis and miRNAs (12-20). There are also other studies on the clinical importance of miRNA gene promoter methylation in PCa (21-25).

The human genome encodes five DNA methyltransferases (DNMTs). Namely, DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L. DNMT1, DNMT3A and DNMT3B are canonical C5 DNMTs that catalyze the addition of methylation markers to genomic DNA (26). Abnormalities in DNMT genes can trigger malignancy, deteriorate prognosis and complicate treatment. DNMT inhibition reduces tumor formation by increasing the expression of tumor suppressor genes and this has resulted in the inclusion of DNMT inhibitors as

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anti-cancer targets (27). The effects of DNMT genes on tumors and therapeutic approaches targeting DNMTs remain a matter of discussion today (28).

Phosphate and tensin homolog (PTEN) gene is a tumor suppressor that regulates cell growth and survival via the phosphoinositol-3 kinase pathway (PI3K) (29). Loss of PTEN expression is observed in a ~70% of PCa patients (30,31). NK3 Homeobox 1 (NKX3.1) is an important homeobox gene for prostatic epithelial specification, proper differentiation and luminal stem cells. Concurrently, this gene is another tumor suppressor gene that regulates the androgen receptor (AR)-related signaling pathway. The loss of PTEN in human prostate cancers may cause a decrease in NKX3.1 expression. Thus, NKX3.1 is a therapeutic target in the treatment of PTEN-deficient prostate cancers (32,33). It has been indicated that NKX3.1 is not expressed in metastatic prostate cancer and may be a valuable biomarker for precisely determining prostatic origin in poorly differentiated metastatic carcinomas (34).

Epigenetic interactions are candidates for new prognostic biomarkers and can be detected by integrating methylation and miRNA linkages (35,36). Epigenetic-based miRNAs (epi-miRNAs) are defined as a new class of miRNAs that suppress enzymes involved in this epigenetic mechanism (36). These miRNAs can directly inhibit enzymes, such as DNMTs and histone deacetylases (HDACs), which are key mediators of epigenetic mechanisms (37). epi-miR29 is the first epi-miRNA identified in lung cancer (36,38). However, the number of experimentally validated epi-miRs involved in these regulatory epigenetic cycles is relatively low (39).

The miR-34 family consists of three members: miR-34a, miR-34b and miR-34c. While miR-34a is encoded with its own transcript, miR-34b and miR-34c share a common primary transcript (40). Since upregulation of the miR-34 family ceases the cell cycle in the G1 phase and leads to cell apoptosis, it can serve a tumor suppressive role (41,42). Vogt *et al* (43) reported the CpG methylation levels of miR-34a and miR-34b/c as being between 40-100% in colorectal, pancreatic, breast, ovarian, urothelial and kidney cancers, and stated that these epi-miRs may have diagnostic value.

miR-148a indicates different expressions in different cancers (44). Hamilton *et al* (45) emphasized that miR-148a is subject to stage-specific regulations in PCa. Yu *et al* (46) concluded that miR-148a may be a tumor suppressing miR, indicating that it is downregulated in gastric cancer. In another study, miR-148a expression was found to be inversely correlated with p27 expression and was downregulated in advanced gastric cancer (47). Other studies have also reported the downregulation of miR-148 in PCa (48,49). By contrast, miR-148a was found to be a prognostic onco-miR capable of regulating EGFR and apoptosis in glioblastoma by targeting mitogen-inducible gene 6 (MIG6) and proapoptotic bim (BCL2L11) (50). It was also found that increased miR-148a-3p in serum can be a potential biomarker in PCa (51). Szczyrba *et al* (52) reported the upregulation of miR-148a (~5-fold increase) in PCa.

miR-152 is a member of the miR-148/152 family. miR-152 has been indicated to act as a tumor suppressor in human cancers (53-55). miR-148a and miR-152 have been shown to target the 3'-UTR region of DNMT1 (56). In another study, miR-152 was reported to suppress PTEN expression by targeting the 3'-UTR region of PTEN (57). Contrary to studies reporting downregulation of miR-152, there are other studies in which miR-152 is upregulated in colorectal, lung and breast cancers (58) and in prostate cancer (6,59).

The miR-200 family has been indicated to facilitate oncogenic activity via inhibition of the tumor suppressor Ras Association Domain Family Member 2 (RASSF2) in colon cancer (60). The oncogenic effects of miR-200 family were also identified in bladder cancer (61). Moreover, in murine lung adenocarcinoma cells, the miR-200 family has also been reported to act as a tumor suppressing miR, inhibiting epithelial-mesenchymal transition (EMT) and metastasis (62). Furthermore, tumor suppressing miR negatively regulates the Rho/Rho-associated kinase ROCK signaling pathway of miR-200 family in hepatacellular carcinoma (63).

In a preliminary study where miR-34b/c, miR-148a and miR-152 were identified as prognostic onco-miR biomarker candidates in PCa, it was found that these epi-miRNAs were correlated with the clinicopathological (PSA, GS and TNM-Staging) characteristics of the patients (25). It was also reported that miR-200a/b acted as an onco-miR in the early stage of PCa and a tumor suppressing miR in the advanced metastatic stage. To the best of our knowledge, comparative multiple analyzes of miR-34b/c, 148a, 152 and 200a/b epi-miRNAs with DNMTs, PTEN and NKX3.1 are not yet available in the literature, which signifies the novelty of the current study. With regards to the aformentioned information, the aim of the present study was to determine the epigenetic interaction between the target genes DNMT1, DNMT3a, DNMT3b, PTEN and NKX3.1, and miR-34b/c, 148a, 152 and 200a/b epi-miRNAs in patients with prostate cancer. The aim was also to determine the miRNA-mRNA regulatory interaction links of each gene and find their possible roles in diagnostic and therapeutic prognosis in PCa.

Materials and methods

Collection of clinical samples. The present study was performed with blood samples taken from 25 healthy patients in the control group, 25 patients with PCa and 40 patients with metastatic prostate cancer (Met-PCa), which were collected with the approval of Gazi University Faculty of Medicine Ethics Committee (Ankara, Turkey; approval no. G.Ü.E.T-686). Whole blood samples included in the study were obtained from patients who applied to the Urology Clinics of the Gazi University Hospital and the Hacettepe University Hospital (Ankara, Turkey). Informed consent was provided by all patients. The age ranges of the patients were 54-70 years for PCa, 40-85 years for metastatic cases and 41-59 years for the control group. Inclusion criteria in the present study were as follows: i) The 65 PCa and Met-PCa patients had not undergone any therapies or surgeries related to PCa; and ii) the 25 healthy controls have not had a family history of PCa. The individuals included in the patient and control groups did not have any known systemic diseases. The clinicopathological characteristics of the patients and healthy controls are listed in Table I. In the metastatic group, a blood sample from one patient had to be discarded, thus could not be included in the data.

RNA isolation. The blood samples were stored in room temperature and the steps specified in the RNA isolation kit

Table I. Clinicopathological characteristics of the patients and controls.

Characteristic	PCa[a] (n=25)	Met-PCa (n=39)	Control
Median age, years (range)	61 (54-70)	68 (40-85)	50 (41-59)
PSA levels at diagnosis, ng/ml			
<2.5			25
<10	19	0	0
10-20	5	10	0
>20	1	29	0
Gleason score			
6	10	0	0
7	11	0	0
8	3	11	0
9	0	16	0
10	1	12	0
Pathological stage			
T2N0M0	15	0	0
T3N0M0	10	0	0
T3N0/1M1	0	10	0
T3/4N0/1M1	0	24	0
T4N0/1M1	0	5	0

[a]Local/Locally advanced prostate cancer. Met-PCa, metastatic prostate cancer; PSA, prostate-specific antigen.

(miRNeasy Serum/Plasma Kit; Qiagen GmbH) protocol were applied. After adding 200 μl serum and 1/5 of the QIAzole lysis buffer from the kit, vortexing was performed to mix the contents. The resulting mixture was kept at room temperature for 5 min. Subsequently, 200 μl chloroform was added and vortexing was carried out for 15 sec. After 2-3 min, the solution was centrifuged at 12,000 x g and 4˚C for 15 min. The top phase containing RNA was transferred to another tube (~600 μl). In total, 1.5 volumes of 100% ethanol was added and then the solution was mixed thoroughly by pipetting up and down several times in the RNeasy MinElute spin column within the 2 ml collection tube; and centrifuged at 8,000 x g for 15 sec at room temperature. The flow-through (containing QIAzole lysis reagent or buffer RWT) was discarded and the spin column was reused in the new collection tube. Then 700 μl wash buffer RWT was added and the tube was centrifuged at 8,000 x g for 15 sec at room temperature. The flow-through was discarded and the spin column was again reused in the new collection tube. Another washing buffer of about 500 μl RPE was added and the tube was centrifuged at 8,000 x g for 15 sec at room temperature. The flow-through was once again discarded and the spin column was placed in the new collection tube. Subsequently 500 μl of 80% ethanol was added into the RNeasy MinElute spin column and the tube was centrifuged at 8,000 x g for 2 min at room temperature. After centrifugation, the RNeasy MinElute spin column was removed and placed in the new collection tube for the last time, such that the spin column would not come into contact with the flow remaining under the collection tube. The spin column with an open the lid was centrifuged at 14,000 x g 4˚C for 5 min to remove ethanol and was then dried. The dried spin column was transferred to a new 1.5 ml collection tube; 14 μl RNase-free water was added to it and was centrifuged at 14,000 x g 4˚C for 1 min. The samples were stored at -20˚C until they were studied.

cDNA synthesis and fluorimetric measurement. The purity and concentrations of the obtained RNAs were measured by colibri microvolume spectrophotometric method (Colibri LB915, Titertek-Berthold). The protocol of the cDNA synthesis kit (QuantiNova reverse transcription kit; Qiagen GmbH) was followed. The total volume was adjusted to 15 μl by adding 13 μl total RNA and 2 μl qDNA separation mixture. After 2 min at 45˚C in the thermal cycler, the device was stopped and the samples were placed on ice. Afterwards, 4 μl of reverse transcription master mix and 1 μl of reverse transcription enzyme were added into each tube and the final volume was brought to 20 μl. The cDNA was synthesized by setting the tubes in the Thermal Cycler for 3 min at 25˚C, 10 min at 45˚C and 5 min at 85˚C. The samples were stored at -20˚C until they were studied.

Quantification of mRNA levels using quantitative (q)PCR. Expression levels of a total of six genes, one being a reference gene, were measured in the present study. The primers used are listed in Table II. The necessary steps were followed according to the manufacturer's instructions: A total of 15 μl of mixture was prepared; each sample containing 4.5 μl water, 0.5 μl of the primer pair, 10 μl of the SYBR master mix (Bioline sensiFAST SYBR 2x; Bioline Reagents Ltd.). The final volume was made up to 20 μl by adding 5 μl of cDNA. Next, 40 cycles of qPCR was performed using the Qiagen

Table II. Targeted primer sequences for reverse transcription-quantitative PCR.

Gene	Primer sequence
β-actin	F: 5'-GAAGATCAAGATCATTGCTCCT-3'
	R: 5'-ACTCGTCATACTCCTGCTT-3'
DNMT1	F: 5'-ACCACCATCACATCTCATT-3'
	R: 5'-GTCTAGCAACTCGTTCTCT-3'
DNMT3a	F: 5'-CGGAACATTGAGGACATCT-3'
	R: 5'-GTACTGGTACGCACACTC-3'
DNMT3b	F: 5'-GAAGATCAGAGCCGAGAACAA-3'
	R: 5'-TCAAAGAGAGGGTGGAAGGA-3'
PTEN	F: 5'-TTAGACTTGACCTATATTTATCCA-3'
	R: 5'-GCGGTGTCATAATGTCTT-3'
NKX3.1	F: 5'-AGAGACCGAGCCAGAAAGG-3'
	R: 5'-GCTTCTGCGGCTGCTTAG-3'

F, Forward; R, Reverse; DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog; NKX3.1, NK3 Homeobox 1.

rotor-gene-Q instrument (Qiagen GmbH) and the thermo-cycling conditions were as follows: Denaturation at 95°C for 1 min, annealing at 61°C for 30 sec and extension at 72°C for 1 min. Experiments were conducted in triplicate. β-actin gene was used as the housekeeping gene.

Statistical analysis. Intergroup mRNA expression levels were analyzed with the Kruskal Wallis-H test using SPSS 22.0 (IBM Corp.). After the Kruskal Wallis-H analysis, complementary benchmarking techniques were used to determine which groups caused the significant difference. Comparative analysis of three different groups in pairs (PCa vs. control, Met-PCa vs. control and PCa vs. Met-PCa) was conducted and confirmed Tamhane's T2 post hoc test. The statistical data are consistent and there were not any statistical changes in the present results. In order to examine the correlation between patient groups and certain clinicopathological parameters (Gleason Score, PSA level and TNM staging), multinomial logistic regression analysis was performed. Finally, linear regression analysis was performed to reveal the association between epi-miRNAs and their target genes. P<0.05 was considered to indicate a statistically significant difference.

Results

The expression levels of DNMT1, DNMT3a, DNMT3b and PTEN were analyzed and statistically significant differences were found between the PCa and Met-PCa groups, and the control group (P<0.05; Fig. 1A and B). However, the expression level of NKX3.1, which is one of the target genes of the investigated miRNAs, did not exhibit a significant difference between the PCa and Met-PCa groups, and the control group (P>0.05; Fig. 1A and B). It is worth emphasizing that there were statistically significant differences between the PCa-control and Met-PCa-control groups, but no significant difference was found between the PCa and Met-PCa groups (P>0.05). Since

NKX3.1 expression level did not differ between groups, it was not included in the clinicopathological evaluation of the present study, and hence in complementary comparative analyzes. When the PCa and Met-PCa groups were compared with the control group, a significant increase in expression level of the DNMT1 gene was found to be ~5-fold in the PCa group and ~6-fold in the Met-PCa group (P<0.001; Fig. 1A). Again, when the PCa and Met-PCa groups were compared with the control, DNMT3a decreased ~5-fold in the PCa group and ~2-fold in the Met-PCa group. In addition, the expression level of DNMT3b decreased ~3-fold in the PCa group and ~2-fold in the Met-PCa group (P<0.01; Fig. 1A). When compared to the control, while decreased expression in DNMT3a and DNMT3b genes was significant in both PCa and Met-PCa groups, decreased expression in the PTEN gene was found to be significant only in the Met-PCa group (P<0.01; Fig. 1A). Moreover, the expression level of PTEN decreased ~2-fold in the PCa group compared with the control, and it decreased ~4-fold in the Met-PCa group (Fig. 1A). In terms of NKX3.1 expression level, no significant difference was found between any groups (P>0.05; Fig. 1A and B). The evaluation results of the patients classified according to their clinicopathological characteristics are provided in Table III (PSA), Table IV (GS) and Table V (TNM). A significant difference was found only in DNMT1 in both groups (P<0.01).

According to the results of linear regression analysis between epi-miRNAs and their target genes, in the PCa group, there was a statistically significant association between miR-34b and DNMT1/DNMT3b; between miR-34c/miR-148a and all target genes studied; between miR-152 and DNMT1/DNMT3b/PTEN; and between miR-200a/b and DNMT1 (P<0.05) (Table VI). In the same patient group, a highly significant association was observed between all miRNAs investigated and DNMT1 (P<0.01; Table VI). Since an inversely proportional interaction between DNMT1 and tumor suppressor genes has been demonstrated, the effects of DNMT1 on PTEN (a tumor suppressor gene) were investigated. In the present study, while there was no statistically significant difference between PTEN and DNMT1 in the PCa and control groups (P>0.05), a highly significant difference was found between PTEN and DNMT1 in the Met-PCa group (P<0.0001) (Table VI). In the Met-PCa group, miR-148a, miR-152 and miR-200b indicated a statistically significant association with all target genes (P<0.01; Table VI). In the same group, miR-34b, miR-34c and miR-200a did not indicate a statistically significant association with the target genes (P>0.05; Table VI). In the control group, no statistically significant association was identified between epi-miRNAs and target genes (P>0.05; Table VI). The present findings indicate a significant association between epi-miRNAs and target genes, which are presented in Fig. 2. It is worth noting that the miRNAs can regulate and interaction with numerous mRNAs.

Discussion

miRNA and epigenetic regulators control expression of protein-coding genes. A specific group of miRNAs, defined as epi-miRNAs, and epigenetic regulators form a powerful network of reciprocal expressions and interactions. These
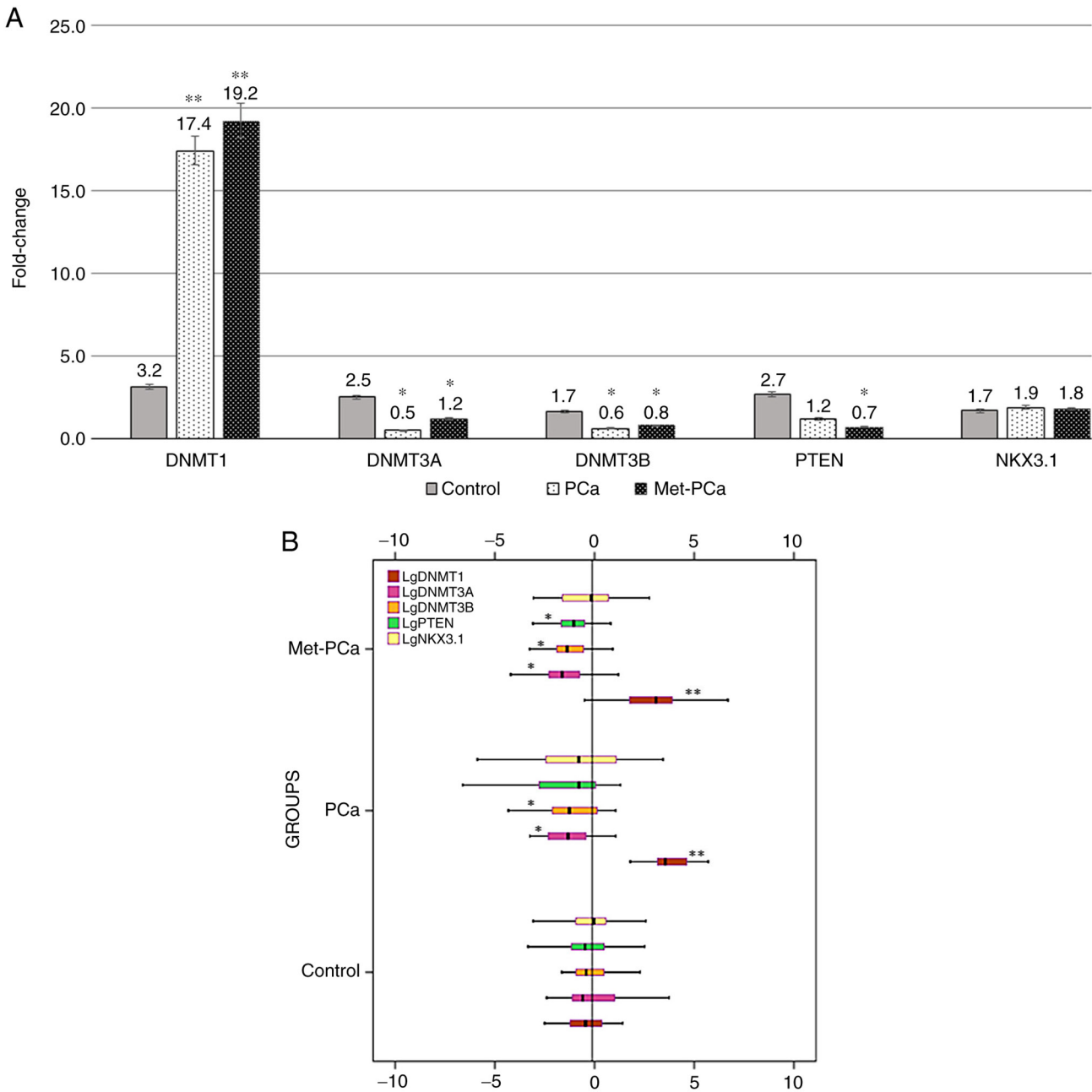
Figure 1. (A) Fold-changes in mRNA expression levels of DNMT1, DNMT3a, DNMT3b, PTEN and NKX3.1 in the healthy group and in patients with PCa and Met-PCa. (B) The box plot of DNMT1, DNMT3a, DNMT3b, PTEN and NKX3.1 in terms of gene expression levels. Mean reference line was -0.1. Values are expressed as the mean ± SEM from triplicate groups. *P<0.01; **P<0.001 vs. Control. DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog; NKX3.1, NK3 Homeobox 1; PCa, prostate cancer; Met-PCa, metastatic prostate cancer.

genetic circuits are regulated by a double-negative feedback loop, in which a miRNA inhibits an epigenetic regulator and is subsequently suppressed by the same regulator (37,64). The epi-miRNA circuit, which is sensitive to the cellular environment, serves a 'transition switch' role, providing strong stability to random cell passages in homeostatic situations. Reale *et al* (39) showed that miRs enhance their regulatory potential by interacting with epigenetic regulatory genes. They also found that the same miR behaves as an epi-miR in a particular cell line, but may not indicate any epigenetic effects in another cell line. For this reason, the 'epi-miRNome' is significant for the successful personalized treatment of cancer. Epi-miRs are particularly noteworthy to elucidate the

etiopathogenesis of the disease and the similarity between cells in the same tissue and/or cells derived from the same type of tumors. In this respect, the present study has the potential to shed light on the follow-up and treatment prediction of prostate cancer.

Increased mRNA expression of DNMT1 has been indicated in prostate cancer and benign prostate epithelial cell lines (65). Progressively increasing expression of DNMT1 is associated with urothelial carcinogenesis in the development and precancerous stages of bladder nodular invasive carcinomas (66,67). In the present study, a significant increase in the expression level of the DNMT1 gene was observed in the PCa and Met-PCa groups, and a significant decrease in

Table III. Association between PSA levels and expression levels of DNMT1, DNMT3a, DNMT3b and PTEN.

A, DNTM1

Value	PCa PSA values (ng/ml)		Met-PCa PSA values (ng/ml)	
	<10	≥10	≤20	>20
FC	6.08	19.56	6.69	22.95
P-value	0.014[a]	0.005[a]	0.001[a]	0.0001[a]
Mean ± SD	19±0.693	6±0.492	23±0.504	7±0.500

B, DNMT3a

Value	PCa PSA values		Met-PCa PSA values	
	<10	≥10	≤20	>20
FC	0.59	0.26	1.90	0.83
P-value	0.371	0.146	0.154	0.817
Mean ± SD	0.6±1.099	0.3±1.429	2±0.746	0.8±0.697

C, DNMT3b

Value	PCa PSA values		Met-PCa PSA values	
	<10	≥10	≤20	>20
FC	0.69	0.41	1.15	0.67
P-value	0.805	0.838	0.534	0.092
Mean ± SD	0.7±1.587	0.4±1.776	1.1±1.229	0.7±1.199

D, PTEN

Value	PCa PSA values		Met-PCa PSA values	
	<10	≥10	≤20	>20
FC	0.75	0.47	1.04	1.58
P-value	0.955	0.874	0.636	0.806
Mean ± SD	0.8±0.957	0.5±1.096	1±0.422	1.6±0.423

FC values were calculated relative to control. PSA levels of controls were <2.5 ng/ml. [a]$P<0.05$ vs. control. Multinomial logistic regression analysis was performed. FC, fold-change; SD, standard deviation; DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog; PSA, prostate-specific antigen.

DNMT3a, DNMT3b and PTEN gene expression levels was also noted. There are many studies reporting that the expression of the DNMT1 enzyme increases in cancer, as well as studies with the opposite results. One of the reasons for this discrepancy is that DNMT1 upregulation is not a secondary effect of increased cancer cell proliferative activity, but may also be caused by mutations in this enzyme (28,68). The decrease in PTEN gene expression and increase in DNMT1 expression in both patient groups are consistent with the literature. These results suggest that DNMT1 may cause a decrease in the expression of PTEN by causing hypermethylation of

its promoter. In the present study, DNMT1, which was found to be highly expressed, downregulated PTEN expression; and the antagonistic interaction between DNMT1-PTEN was found to be significant only in the Met-PCa group. This result suggests that DNMT1 primarily regulates the PTEN gene at the metastatic stage, and DNMT1/PTEN relative expression may be associated with a less favorable prognosis. Furthermore, it was concluded in the present study that high DNMT1 expression combined with low PTEN expression may represent prognostic markers for overall survival in patients with Met-PCa. This result suggests that the PTEN suppressor

Table IV. Associations between the Gleason Scores and expression levels of DNMT1, DNMT3a, DNMT3b and PTEN.

A, DNTM1

	PCa Gleason Scores				Met-PCa Gleason Scores		
Value	6	7	8	10	8	9	10
FC	15.41	18.83	15.16	24.74	17.75	18.29	21.74
P-value	0.003[a]	0.003[a]	0.006[a]	0.001[a]	0.0001[a]	0.0001[a]	0.0001[a]
Mean ± SD	15±0.496	19±0.578	15±0.778	25±1.817	18±0.566	18±0.49	22±0.524

B, DNMT3a

	PCa Gleason Scores				Met-PCa Gleason Scores		
Value	6	7	8	10	8	9	10
FC	0.53	0.52	0.53	0.33	0.30	1.89	2.58
P-value	0.36	0.241	0.215	0.433	0.22	0.65	0.43
Mean ± SD	0.5±1.099	0.5±1.176	0.5±1.482	0.3±5.274	0.3±0.774	1.9±0.691	2.6±0.709

C, DNMT3b

	PCa Gleason Scores				Met-PCa Gleason Scores		
Value	6	7	8	10	8	9	10
FC	0.63	0.64	0.52	0.56	0.34	1.18	1.48
P-value	0.854	0.882	0.969	0.65	0.16	0.13	0.26
Mean ± SD	0.6±1.491	0.6±1.504	0.5±1.748	0.6±5.56	0.3±1.263	1.2±1.197	1.5±1.206

D, PTEN

	PCa Gleason Scores				Met-PCa Gleason Scores		
Value	6	7	8	10	8	9	10
FC	0.72	0.68	0.73	0.44	0.41	2.00	0.74
P-value	0.885	0.929	0.932	0.983	0.91	0.99	0.94
Mean ± SD	0.7±0.917	0.7±0.949	0.7±1.211	0.4±2.278	0.4±0.452	2±0.404	0.7±0.408

[a]$P<0.01$. Multinomial logistic regression analysis was performed. FC, fold-change; SD, standard deviation; DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog.

gene may be the target of other epigenetic regulatory enzymes in addition to DNMT1 in the metastatic process, leading to multiple changes, such as loss of heterozygosity and allelic deletion. In other words, the decrease in PTEN accompanying the increase in DNMT1 expression level is perhaps rare in early-stage prostate cancer, but may be encountered frequently with tumor progression. In parallel to the study by Qi *et al* (69) on bladder cancer, the present study demonstrated that the DNMT1 expression indicated a significant association with clinicopathological features (PSA, GS and TNM) in the PCa and Met-PCa groups. Statistical significance of only the DNMT1 gene in terms of all clinicopathological stages proves that this gene serves an active role in both the early stage and the advanced stage of PCa. The present result indicates the potential of DNMT1 as a therapeutic target and candidate for a biomarker. Since a literature search did not return any results for studies that associate DNMTs with prostate cancer according to clinicopathological features, it wasn't possile to make a comparison in terms of the latter part of the present results. It has been indicated that DNMT3b (70) and DNMT3a (71) can be potential targets in developing appropriate diagnostics and therapeutics in breast cancer. Ma *et al* (72) noted that DNMT1- and DNMT3a-targeting tumor suppressor genes in pituitary adenomas were upregulated, and were associated

Table V. Associations between TNM staging and the expression levels of DNMT1. DNMT3a. DNMT3b and PTEN.

A, DNTM1

Value	PCa TNM classification		Met-PCaTNM classification		
	T2N0M0	T3N0M0	T3N0/1M1	T3/4 N0/1M1	T4 N0/1M1
FC	18.31	26.04	10.41	24.66	30.55
P-value	0.002[a]	0.001[a]	0.0001[a]	0.0001[a]	0.0001[a]
Mean ± SD	18±0.472	16±0.618	10.4±0.581	24.66±0.517	10.6±0.508

B, DNMT3a

Value	PCa TNM classification		Met-PCa TNM classification		
	T2N0M0	T3N0M0	T3N0/1M1	T3/4 N0/1M1	T4 N0/1M1
FC	0.55	0.53	0.28	2.29	1.37
P-value	0.255	0.374	0.071	0.768	0.805
Mean ± SD	0.5±1.117	0.5±1.156	0.3±0.812	2.3±0.714	1.4±0.833

C, DNMT3b

Value	PCa TNM classification		Met-PCa TNM classification		
	T2N0M0	T3N0M0	T3N0/1M1	T3/4 N0/1M1	T4 N0/1M1
FC	0.74	0.50	0.43	1.31	0.96
P-value	0.889	0.804	0.451	0.127	0.261
Mean ± SD	0.7±1.46	0.5±1.518	0.4±1.274	1.3±1.206	1±1.32

D, PTEN

Value	PCa TNM classification		Met-PCa TNM classification\		
	T2N0M0	T3N0M0	T3N0/1M1	T3/4 N0/1M1	T4 N0/1M1
FC	0.76	0.62	2.83	0.61	0.49
P-value	0.953	0.678	0.389	0.762	0.792
Mean ± SD	0.8±0.932	0.6±0.97	2.8±0.499	0.6±0.468	0.5±0.527

[a]$P<0.01$. Multinomial logistic regression analysis was performed. FC, fold-change; SD, standard deviation; DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog.

with aggressive tumor behavior and high methylation. It has also been demonstrated that DNMT1 is positively correlated with tumor size, clinical stage, histological grading, lymph node metastasis, vascular invasion, recurrence and prognosis in renal cell carcinoma (73). In urological cancers, DNMTs have also been investigated (74-76). The vast majority of studies have highlighted the importance of the methylation pattern in the discovery of new anticancer therapeutics. Bowen *et al* (33) showed that loss of NKX3.1, a gatekeeper suppressor, increased prostate epithelial cell proliferation in mice, but NKX3.1 mRNA expression was not affected by PTEN. Therefore, it was suggested that the interaction of tumor suppressors PTEN

and NKX3.1 may be responsible for the loss of PTEN. The loss of function of NKX3.1 caused inflammation-induced prostate cancer through abnormal cellular plasticity and impairment of cellular differentiation in a mouse model (77). In the present study, no significant difference was found in NKX3.1 gene expression between any groups and the control. The reason thelatter result differed from those in the literature may be due to the fact that human serum instead was used in the present study rather than mouse tissue.

Majid *et al* (42) investigated prostate cancer in a xenograft mouse model and found that miR-34b inhibited cell proliferation, colony formation and migration/invasion by directly

Table VI. P-values between the expression levels of epi-miRNAs and expression levels of DNMT1, DNMT3a, DNMT3b and PTEN.

A, PCa

| Genes | P-values of Epi-miRNAs | | | | | | Genes |
	miR-34b	miR-34c	miR-148a	miR-152	miR-200a	miR-200b	DNMT1
DNMT1	0.004[a]	0.034[a]	0.001[a]	0.009[a]	0.011[a]	0.002[a]	
DNMT3a	0.37	0.01[a]	0.042[a]	0.156	0.67	0.951	
DNMT3b	0.043[a]	0.023[a]	0.007[a]	0.022[a]	0.127	0.305	
PTEN	0.058	0.037[a]	0.008[a]	0.004[a]	0.918	0.835	0.089

B, Met-PCa

| Genes | P-values of Epi-miRNAs | | | | | | Genes |
	miR-34b	miR-34c	miR-148a	miR-152	miR-200a	miR-200b	DNMT1
DNMT1	0.951	0.127	0.016[a]	0.002[a]	0.979	0.001[a]	
DNMT3a	0.856	0.283	0.018[a]	0.004[a]	0.404	0.015[a]	
DNMT3b	0.057	0.054	0.002[a]	0.007[a]	0.077	0.001[a]	
PTEN	0.881	0.667	0.003[a]	0.004[a]	0.578	0.003[a]	0.0001[a]

C, Control

| Genes | P-values of Epi-miRNAs | | | | | | |
	miR-34b	miR-34c	miR-148a	miR-152	miR-200a	miR-200b	DNMT1
DNMT1	0.609	0.624	0.812	0.211	0.089	0.141	
DNMT3a	0.107	0.223	0.942	0.054	0.199	0.763	
DNMT3b	0.06	0.051	0.578	0.261	0.05	0.86	
PTEN	0.907	0.097	0.951	0.78	0.167	0.272	0.479

[a]$P<0.01$. Linear regression analysis was performed. miR, microRNA; DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog.

targeting Akt and its downstream genes, and induced apoptosis by stopping the cell cycle at G0/G1. In the same study, the increased expression of miR-34b was reported to downregulate DNMTs and HDACs by inducing partial demethylation and active chromatin modifications. Shiina *et al* (78) reported that downregulation of miR-34b upregulated androgen receptor (AR) in African-Americans with PCa, pointing to a potential association between race and tumorigenesis. Conversely, these low expressions levels possibly stemming from allelic deletions and/or loss of heterozygosity should not be overlooked. Contrary to the above studies, Chamani *et al* (79) found that expressions of DNMT1, DNMT3a and DNMT3b were down-regulated in hepatocellular cancer cells treated with dendrosomal nanocurcumin, while miR-34a, -34b and -34c were upregulated. It was demonstrated that there is an inverse proportion between the expression level of miR-34 and DNMTs. In a preliminary study (25), it was found that the percentages of promoter methylation of miR-34b and miR-34c decreased in the PCa and Met-PCa groups, and this

hypomethylation resulted in an increased expression level of miR-34b/c. Furthermore, high miR-34b/c levels were associated directly with PSA, GS and TNM staging. In the current study, parallel to the literature, an inversely proportional relationship was identified between the highly expressed miR-34c and target genes DNMT3a, 3b and PTEN. In addition, it was found that high levels of DNMT1 were associated with high levels of miR-34b and miR-34c in the PCa group. We hypothesized that the high level of miR-34b/c may stem from the mutant DNMT1. However, the conclusion emphasizing that high DNMT1 level was associated with high miR-34b and miR-34c levels was not consistent with the findings of Majid *et al* (42). The reason for this may be the pleiotropic role of miR-34b, as well as the different tissue sources included in the study protocol, differences in sample selection, sample size, ethnic composition, mutations in the DNMT1 enzyme and regulations of DNMT1 enzyme with different miRNAs. In the Met-PCa group, no statistically significant difference was found between miR-34b/c and target genes. By increasing
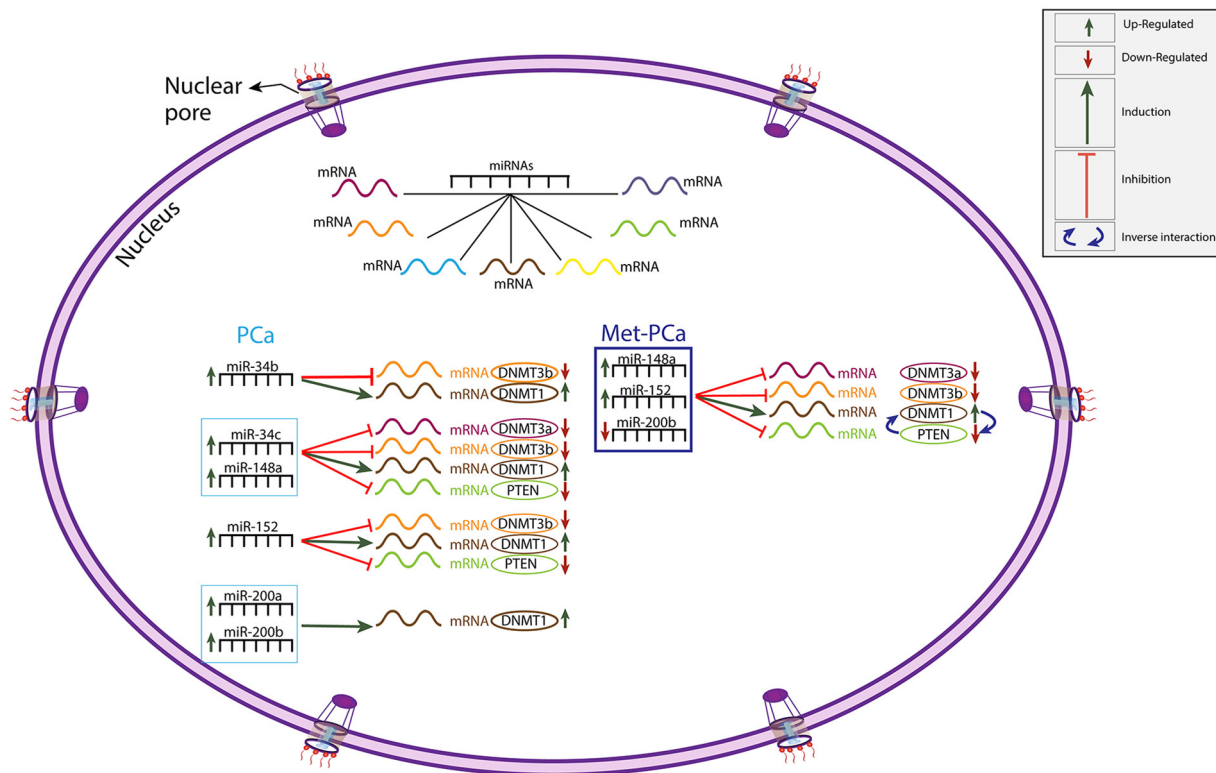
Figure 2. miRNAs regulate numerous mRNAs in PCa. The six key epi-miRNAs and target epigenetic signature genes they regulate in the progression of PCa. miRNA/miR, microRNA; DNMT, DNA methyltransferase; PTEN, phosphate and tensin homolog; PCa, prostate cancer; Met-PCa, metastatic prostate cancer.

the expression of DNMT1 in the early stages of PCa and suppressing the PTEN gene, miR-34c may represent a potential diagnostic and prognostic target of a new regulatory pathway: miR-34c-DNMT1-PTEN, although this would have to be confirmed in further *in vivo* studies.

Sengupta *et al* (80) demonstrated that in hormone-resistant prostate cancer cell lines, ectopic expression of miR-148a induces apoptosis by suppressing DNMT1 expression. They also noted that there was an antagonistic relationship between miR-148a and DNMT1 expression levels. miR-148a regulated the expression of DNMT genes by binding to the coding region of DNMT1 and DNMT3b (56,81). Braconi *et al* (56) identified that the precursors of miR-148a and miR-152 increased the expression of methylation-sensitive tumor suppressor genes Rassf1a and p16INK4a by decreasing DNMT1 protein expression in human cholangiocarcinoma cell line. Hamilton *et al* (45) found that miR-148a targets both oncogenes and tumor suppressors, such as PTEN in PCa, and its expression level varies according to the stage of the disease. Moreover, Li *et al* (44) indicated that miR-148a-3p was upregulated in certain cancers and downregulated in others. miR-148a was upregulated in glioblastoma (82) and in osteosarcoma (83), which reflects the aggressiveness of the cancers. In another study, miR-148a induced activation of the phosphatidylinositol 3-kinase (PI3K) signaling pathway in osteosarcomas by targeting PTEN (84). Furthermore, miR-148a increased β-catenin expression by inhibiting PTEN expression (85). Dybos *et al* (51) investigated miR-148a-3p in PCa and reported that high miR-148a-3p was much more than a general marker of imbalance, such as infections in the body. The aforementioned studies support our hypothesis,

drawing attention to the potential role of miR-148a overexpression in PCa development. Consistent with the literature, in the PCa and Met-PCa groups, it was revealed that hypomethylated miR-148a interacted inversely with target genes DNMT3a, 3b and PTEN; however, unlike in the literature, it interacted directly with DNMT1. It was revealed that high DNMT1 expression levels were associated with high miR-148a level in both patient groups. This result suggests that the expression level of miR-148a may have been increased due to the mutant DNMT1 gene.

A highly significant association between DNMT1 and PTEN genes was identified in the Met-PCa group. A possible mechanism for the increased expression of miR-148a is the hypomethylation of the miR-148a host gene promoter that stimulates transcription of this miRNA due to its onco-miR nature. It was revealed that miR-148a downregulated PTEN expression by targeting DNMT1. PTEN activity, in addition to epigenetic silencing and mutations, can also be regulated in a variety of ways, such as transcriptional inhibiting, abnormal protein localization and post-translational modifications. The present findings revealed that the DNMT1 gene was in the upstream cascade of PTEN and it downregulated PTEN via promoter hypermethylation that promoted metastasis of PCa. The significance of the linkage between DNMT1 and PTEN genes in the metastatic stage of the disease suggests that tumor suppressor gene deletion may be involved in the metastatic state.

Another non-coding RNA that serves key roles in PCa pathogenesis is miR-152. It has been reported that the expression level of miR-152 was significantly decreased in PCa tissues with high Gleason Score (53,54). Theodore *et al* (54) reported that expression of miR-152 decreased due to the high

methylation profile in PCa cell lines, in contrast to increased expression of DNMT1. They also noted that miR-152/DNMT1 epigenetic regulation may serve a key role in aggressiveness of PCa tumors, particularly in African-American populations. In another study, the expression level of miR-152 decreased in PCa tissue due to the high methylation profile (86). In contrast to the study by Ramalho-Carvalho *et al* (86), high plasma miR-152 levels have been proposed as a diagnostic biomarker for other cancers such as lung, colorectal and breast cancer (58). Matin *et al* (6) reported that overexpression of miR-152-3p increased the proliferation and migration of prostate cancer cells. Moya *et al* (59) identified one of the four plasma miRNAs (a highly expressed miR-152) that has the potential to improve the diagnostic power of the existing PSA biomarker for PCa. In a preliminary study (25), we found that the increase in the expression of miR-152 as a result of promoter hypomethylation was directly proportional to PSA, GS and TNM staging in both PCa and Met-PCa groups. Our results did not support the finding of the study by Zhu *et al* (53), Theodore *et al* (54) and Ramalho-Carvalho *et al* (86). The reasons for this are that detected levels of miRNA can vary significantly, especially depending on the extraction methodology applied in serum samples (pre-extraction steps such as sample collection, sampling, processing and storage, and internal controls used), sample size, tissue type studied and racial differences. Moreover, since miRNA secretion is a selective process, the circulatory levels are not a true reflection of intracellular levels (87). In addition, due to the unknown effects of the coagulation process on extracellular miRNAs in the blood, levels of miRNA concentrations in serum rather than in plasma were examined. Therefore, comparisons among miRNA irregularities in different samples became even more difficult. For example, while miR-152 was upregulated in serum (88), it was downregulated in tissue samples (89) from patients with bladder cancer. The discrepancy might be attributed to the metastatic role of miR-152, known as exosomal-miR or metasta-miR, possibly via exosomal escape (90,91). Exosomes derived from prostate cancer cells contribute to the chemo-resistance of cancer (91). In the present study, high levels of miR-152 were further upregulated due to the resistance in the metastatic stage, even resulting in loss of PTEN. Also, certain miRNAs are released by tumor cells as argonaute (AGO)-bound complexes or from dead cells in apoptotic bodies; thus, one source of miRNAs is tumor cells (92). Perhaps the source of high levels of miR-152s in the present study may have been PCa cells. Chen *et al* (58) reported similar results. They investigated the plasma values of onco-miR-21 and the tumor suppressor miR-152 and found that the expression levels of both miRs were increased. In the PCa and Met-PCa groups from the current study, it was demonstrated that upregulation of miR-152 was significantly associated with upregulation of DNMT1, and with downregulation of DNMT3b and PTEN. The current findings were consistent with a nasopharyngeal carcinoma study by Huang *et al* (57), who showed miR-152 inhibited PTEN expression by targeting the 3'UTR of the PTEN mRNA. The present study also revealed an inversely proportional relationship between miR-152 and PTEN expression levels in both groups. Moreover, a highly significant association was demonstrated between miR-152 and DNMT1 expression in the Met-PCa group, as it was between miR-148a

and DNMT1. The present result suggests that mutant DNMT1 gene might increase the expression level of miR-152 and this causes PTEN gene to be silenced by methylation. To the best of our knowledge, the miR-152-DNMT1-PTEN pathway has not yet been investigated in PCa, therefore, it was not possible to directly compare the current results with other data from the literature.

Downregulation of the miRNA-200 family is a potential tumor-suppressor in PCa (93). Low miRNA-200 family expression causes upregulation of zinc finger E homeobox binding transcription factors (ZEB)1 and ZEB2, which are best known for their role in sustaining EMT (94). miR-200a and b cause the growth of endometrial cancer cells *in vitro*, by suppressing PTEN gene expression (95). In another study on endometrial cancer, miR-200a, miR-200b and miR-429 were found to be onco-miRs targeting PTEN (96). Suo *et al* (97) reported that miR-200a, which was overexpressed in the ovarian cancer tissue and cell lines, inhibited PTEN expression and had a carcinogenic effect for this type of cancer. As is known, target genes of miR-200a/b include DNMT3a, DNMT3b and DNMT1 (71). The downregulation of miR-200b and miR-200c-mediated DNMTs increased the chemotherapeutic sensitivity of ovarian cancer cells *in vivo* by reversing cisplatin resistance (98). In a breast cancer cell line, Zeng *et al* (99) identified that flap endonuclease 1, which is an important gene in DNA replication, excision repair and telomere maintenance, caused DNMT1 upregulation, which was associated with downregulation of miR-200a. The miR-200 family generally behaves in a cancer-specific manner, their expression is increased in certain cancers but decreased in other cancers. Our precious study demonstrated the pleitropic effect of miR-200a/b family in local/locally advanced and metastatic PCa (25). An increase in miR-200a and miR-200b expression levels due to promoter hypomethylation was observed in the local/locally advanced PCa group; and low expression levels due to promoter hypermethylation were observed in the metastatic group. In other words, it was determined that miR-200a/b behaves as an onco-miR in the early stage of PCa, but as a tumor suppressor-miR in the advanced metastatic stage. In the current study, it was determined in patients with Met-PCa that there was a highly significant association between all target genes and downregulated miR-200b, as well as between all target genes and upregulated miR-148a and miR-152. In the metastatic group, it was indicated that miR-200b, which is downregulated as a result of hypermethylation in its promoter, interacted synergistically with target genes of DNMT3a, DNMT3b and PTEN, but antagonistically with DNMT1. In the PCa patient group, there was a significant association between high miR-200a and high DNMT1 expression. The current findings indicate that downregulated miR-200b in Met-PCa suppresses the PTEN gene and, conversely, stimulates the DNMT1 gene. In the control group, no significant association was found between any epi-miRNAs and target genes investigated. This result indicates that the altered expression of gene-regulating epi-miRNAs serves an active role in the etiopathogenesis of PCa, and that these epi-miRNAs may represent potential biomarker candidates.

The complex association between miRNAs and epigenetic signature is an important milestone for monitoring

gene expression profiles in cancer. Notably, epi-miR-epi trilateral regulatory feedback associations between miRNAs and epigenetic patterns can target epigenetic modifying enzymes (37). To the best of our knowledge, the present study was the first analyzing the signaling relationship between epi-miRs and target genes, which is associated with prognostic effects of miR-34b/c, miR-148a, mir-152 and miR-200a/b epi-miRNAs and DNMTs, PTEN and NKX3.1, in the local and metastatic stages of prostate cancer development. Examing PCa cell lines for functional role of these miRNAs and target genes in relation to PCa will help clarify this association.

In summary, miR-148a, miR-152 and miR-200b targeting DNMT1/PTEN expression levels may represent powerful and distinctive biomarker candidates, and serve as predictive, prognostic and therapeutic targets, particularly in patients with advanced stage PCa. Further studies are needed to explain the precise detailed interaction of PTEN with DNMT1 in PCa in the pattern of tumor suppressor-miR, onco-miR and/or metasta-miR. The current findings are novel and pioneering in that they highlight the relative and holistic expression profiles of epi-miRNAs and target mRNAs in the diagnostic and prognostic process of PCa. The present results may lead the way for other studies with larger cohorts.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VG and EK conceived, designed and performed the research. EK interpreted experimental data. Statistical analysis was performed by VG. VG and EK confirm the authenticity of all the raw data. Clinical data analysis and sample selection were performed by EK, CYB and SS. EK wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Gazi University Faculty of Medicine, Ankara, Turkey (approval no. G.Ü.E.T-686). All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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