Research Article

Expression of miRNAs in prostate cancer cell lines and prostate epithelial cell lines

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Abstract

Objectives: Prostate cancer (PCa) is among the most frequently diagnosed cancers and a leading cause of cancer-related deaths in men. Although prostate-specific antigen (PSA) testing has become routine in screening and early detection, PSA has high false-positive rates and poorly correlates with disease stage. Consequently, other diagnostic and prognostic markers for PCa are urgently needed. MicroRNAs (miRNAs) modulate gene expression at the transcriptional level and are known to play key roles in various physiological events. Growing evidence indicates that miRNA dysregulation is involved in the initiation and progression of many diseases, including PCa. Various miRNAs have been associated with PCa pathogenesis, suggesting that miRNA expression profiles have potential utility in the prognosis, diagnosis, and treatment of this disease. miRNA expression levels have been investigated in prostate epithelial cells by PCa cell culture.

Methods: The present study compared the expression levels of selected prognostic miRNAs targeting that have been implicated in the pathogenesis of PCa. Using cDNA obtained from the C4-2 human PCa and PNT1a normal prostate epithelial cell lines, miRNA expression levels were quantitatively analyzed via melting curve analysis using the miScript SYBR Green kit in a Rotor-Gene Q real-time polymerase chain reaction (PCR) device.

Results: Reverse transcription quantitative PCR analyses have demonstrated miRNA expression levels in the C4-2 PCa cell line and PNT1a prostate epithelial cell line. MiR-125, -145, -200, and -222 were found to be overexpressed in the PCa cell line (p<0.05), while there were no statistically significant differences in miR-32 expression of miR-21, -26, Let-7, -34, or -221 between PCa cells and PNT1a cells (p>0.05).

Conclusion: Considering the variation in expression level of miRNAs targeting genes responsible for the etiopathogenesis of PCa. Provides information on the use of miRNAs as prognostic markers in PCa.

Keywords: Androgen receptor, microRNAs, prostate cancer

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Prostate cancer (PCa) is one of the most common non-skin malignancies and among the main causes of cancer-related death worldwide [1]. Globally, more than 650,000 men are diagnosed with PCa each year, accounting for approximately 10% of all new cancer cases in men. PCa is the most common solid tissue cancer among men in Western societies. Although the widespread use of the prostate-specific antigen (PSA) test and screening methods have increased the frequency of diagnosis, they have significantly reduced PCa-related metastases and mortality. Several risk factors have been identified in relation to the progression of the disease, but relapse is still reported in up to 30% of PCa patients after radical prostatectomy. Therefore, the molecular mechanisms underlying prostate carcinogenesis must be characterized in order to improve patient survival and the efficacy of available treatment options [1, 2].

MicroRNAs (miRNA) are small, non-protein-coding RNA molecules approximately 18–22 nucleotides in length that regulate gene expression at the translational level. The expression of miRNAs is known to be altered in human malignancies [3]. Changes in miRNA expression levels may be tumor-specific and have been associated with prognosis in some conditions. Studies evaluating miRNA expression profiles in cancer patients have demonstrated differences in miRNA expression not only between normal and tumor tissues but also between primary tumor and metastatic tissues. In addition, overexpressed miRNAs may act as oncogenes by inhibiting tumor-suppressing genes, whereas underexpressed miRNAs can act as tumor-suppressor genes through the negative regulation of oncogenes [3].

Existing evidence indicates that miRNAs modulate the expression of genes involved in the epithelial-mesenchymal transition (EMT), the cell cycle and apoptosis, the androgen receptor (AR) signaling pathway, and the formation of metastasis and cancer stem cells. Studies have shown that miRNAs can be used as biomarkers in the diagnosis of PCa. It has been suggested that evaluating miRNA expression profiles in clinical prostate tissue samples may be useful in detecting cancer, predicting and monitoring cancer prognosis, selecting treatment, and monitoring treatment response [1]. To date, approximately 50 miRNAs have been shown to have dysregulated expression in human PCa (approximately 40 miRNAs were reported to be upregulated and the rest are downregulated [1]. Previous studies have investigated the prognostic value of certain miRNAs in PCa (miR–21, –331, –141, –145, –34, –12–22, –10, –100, –30, –224, –182, –187) as well as studies of miRNAs that target apoptosis and the cell cycle in the pathogenesis of PCa (Let7, miR–1519, –21, –23, –24, –26, –31, –32, –125, –133, –145, –182, –34, –204, –1203, –205, –221, –222) [1, 4, 5].

Several miRNAs target the cell cycle and apoptosis. MiR-221/222 regulates the cyclin-dependent kinase inhibitor p27Kipl. Through suppression of p27Kipl, these miRNAs were shown to promote cell cycle progression and proliferation in PC3, an aggressive PCa cell line [6, 7]. MiR-125b was also shown to stimulate tumor growth by silencing proapoptotic genes. In contrast, let-7a, from the let-7 family of tumor-suppressor miRNAs, inhibits tumor growth by causing cell cycle arrest, and was shown to be downregulated in both PCa tissue and cell lines [8].

EMT is the process by which epithelial cells lose their polarity and cell-cell adhesive characteristics and acquire mesenchymal qualities that promote metastasis and increase invasiveness. Some members of the miR-200 family have the ability to reverse EMT by targeting repressors of E-cadherin. However, while some miR-200 family members were found to be downregulated in PCa progression (miR-205), others were found to be increased in advanced disease [9].

AR signaling also plays a direct role in tumorigenesis and disease progression in PCa. Studies have shown that several miRNAs in the AR signaling pathway are dysregulated (let7, miR–21, –31, –32, –141, –182, –125, –205, –185, –221, –222) [10].

The present study examined the expression levels of selected miRNAs that are believed to play a role in the pathogenesis of PCa, in a cell cancer line (C4-2).

Materials and Methods

This study was conducted jointly by the Faculty of Medicine, Medical Biology and Faculty of Veterinary Medicine, Histology and Embryology departments of Ataturk University and Department of Internal Medicine, The City Hospital in Erzurum, Turkiye.

Cell culture

The C4-2 human PCa cell line and PNT1a normal prostate epithelial cell line (American Type Culture Collection, Manassas, VA, USA) were used as *in vitro* models. Cells were cultured in RRMI medium containing 10% fetal bovine serum, 1% L-glutamine, and 1% PSA in a humidified incubator with 5% CO_2 at 37°C. C4-2 and PNT1a cells were seeded in six-well plates at a concentration of 1.5×10^4 and 2×10^5 cells/well, respectively.

A Transfection Reagent kit was used according to the manufacturer's instructions. Transfected cells were harvested for experiments after culturing for 24–48 h.

miRNA extraction and cDNA synthesis

miRNA was isolated using the miScript RNeasy Mini Kit (Hilden, Germany) as per the manufacturer's instructions. The quality of the isolated miRNA was assessed by spectrophotometric analysis (Maestrogen, Maestro Nano Spectrophotometer, USA). From 2 μg of total RNA, cDNA was synthesized by reverse transcription using the Qiagen miScript II Reverse Transcription Kit (Hilden, Germany) in a Labcycler Thermal Cycler (SenSoquest). The cDNA was diluted and used as a template for real-time Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis.

Quantitative PCR

RT-qPCR was performed using Qiagen miScript SYBR Green PCR kit (Qiagen, Germantown, MD, USA) with miScript Primer Assays (Qiagen SABiosciences, USA). The 25-μL reactions contained 12.5 μL of miScript SYBR Green Master Mix, 1 μL of each primer (forward and reverse), 6.5 μL of DNase/RNase-free distilled water, and 5 μL of cDNA template, and were run at 95°C for 2 min, followed by 40 cycles of 94°C for 15 s and 55°C for 30 s in a Rotor-Disc 72 and Qiagen Rotor-gene Q (Qiagen, Hilden, Germany). *SNORD61* was selected as the reference gene for this study. Reference sequence numbers for all primers were obtained from the GenBank and are shown in Table 1. Ct values were determined using Microsoft Excel and uploaded to the data analysis web tool at http://www.qiagen.com/geneglobe. Data from the normal (PNT1a) and PCa (C4-2) groups were labeled and the manually selected reference genes were used to normalize the C_t values. First, ΔC_t values were calcu-

Table 1. Human gene table for the studied microRNAs

miRNA symbol	Catalog number
miR-125	MS00006629
miR-145	MS00003528
miR-221	MS00009219
miR-222	MS00007609
Let-7	MS00003122
miR-34	MS00003332
miR-200	MS00003752
miR-21	MS00009079
miR-32	MS00003297
miR-26	MS00009219

lated between the genes of interest and the β-actin (*ACTB*) housekeeping gene, then $\Delta\Delta C_t$ was determined by subtracting the ΔC_t value of the PNT1a cell line from that of the C4-2 cell line. Fold-change values were determined as $2^{-\Delta\Delta Ct}$. The data analysis report was exported from QIAGEN web portal at GeneGlobe. All reactions werecarried out in duplicates and expression level differences were calculated using $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Fold-change values <1 were also expressed as fold regulation (−1/fold-change). Fold-regulation values of ≥2 and ≤−2 were accepted as up- and downregulation, respectively. Continuous data were compared using dependent or independent Student's t-test as appropriate. P<0.05 were considered significant.

Results

RT-qPCR analyses of miRNA expression levels in the C4-2 PCa cell line and the PNT1a prostate epithelial cell line showed that miR−125, −145, −200, and −222 were overexpressed in the PCa cell line (p<0.05; Table 2). There were no statistically significant

differences in miR-32 expression of miR−21, −26, Let−7, −34, or −221 between PCa cells and PNT1a cells (p>0.05; Table 2).

Discussion

MiRNAs are involved in a wide range of biological processes and are often dysregulated in cancer. Therefore, it has been proposed that miRNAs may serve as valuable markers in the diagnosis, prognosis, and selection of treatment in PCa. The present study examined expression levels of miRNAs in PCa cell culture (C4-2) and a benign prostate epithelial cell line (PNT1a).

miR-21

MiR-21 is one of the known oncogenic miRNAs associated with PCa. MiR-21 was shown to be overexpressed in PCa and involved in tumorigenesis. It also increases the invasiveness and mobility of PCa cells, thereby promoting invasion and metastasis [11].

Ribas et al. [12] demonstrated direct transcriptional regulation of miR-21 by AR. MiR-21 was one of 16 androgen-responsive miRNAs identified in their study by microarray analysis of AR-positive PCa cell lines, and RT-qPCR analysis showed that miR-21 expression was elevated in human PCa tissue when compared to adjacent healthy tissue. They observed that AR was recruited to the miR-21 promoter region after androgen stimulation and that overexpression of miR-21 promoted tumor growth *in vivo*, even overcoming castration-mediated growth arrest in androgen-dependent tumors. These findings strongly suggested that miR-21 is directly regulated by AR and that miR-21 overexpression imparts castration resistance.

In another study, it was determined that circulating miR-21 level was significantly higher in PCa patients compared to controls and suggested that this parameter was a promising biomarker for identifying PCa patients [13].

We detected no statistically significant difference in miR21-5p expression between the PCa and control cell lines. Although

Table 2. Fold changes from gene assays between the prostate cancer cell line and prostate epithelial cell line for the selected transcripts

miRNAs	AVG ΔCt PCa	AVG ΔCt control	$2^{-\Delta\Delta Ct}$ PCa	$2^{-\Delta\Delta Ct}$ control	p	FoldUp/down PCa/control group
hsa-miR-145-5p	−4.98	−2.43	31.559.447	5.388.934	<0.005	5.86
hsa-miR-200c-5p	−5.60	−2.65	48.502.930	6.276.673	<0.005	7.73
hsa-miR-125b-5p	−4.72	−1.75	26.354.913	3.363.586	<0.005	7.84
hsa-miR-222-3p	−4.74	−2.73	26.722.813	6.634.556	<0.005	4.03
hsa-miR-21-5p	−3.77	−4.42	13.642.158	21.406841	>0.005	−1.57
hsa-miR-221-5p	0.14	0.20	0.907.519	0.870.551	>0.005	1.04
Hsp_miR-26a	−3.64	−3.55	12.466.633	11.712.686	>0.005	1.06
hsa-let-7-5p	−4.37	−4.00	20.677.645	16.000.000	>0.005	1.29
hsa-miR-34c-5p	−0.04	0.08	1.028.114	0.946.058	>0.005	1.09
hsa-miR-32-5p	0.69	3.28	0.619.854	0.102.949	>0.005	1.09
SNORD61	000	000	1.000.000	0.102.949	>0.005	1.00

AVG: Average delta CT value; PCa: Prostate cancer; hsa-miR: 145-5p, 200c-5p, 125b-5p, 222-3p, 21-5p, 221-5p, 26a, let-7-5p, 34c-5p, 32-5p; SNORD61: Housekeeping gene.

miR-21 has been proposed as a widely exploitable cancer-related target, our data suggest that it is not a central player in the onset of PCa and that its single hit is not a valuable therapeutic strategy in PCa.

miR-145

AR signaling seems to be the main target of miRNAs in PCa. MiRNAs have been reported to target AR itself or AR-associated factors by binding to the 3'-untranslated region of messenger RNAs. Coarfa et al. [14] reported that the expression of 12 miRNAs, including miR-145-3p, was suppressed in metastatic PCa.

In another study using next generation sequencing, the expression levels of 945–1248 miRNAs in a metastatic castration-resistant PCa (CRPC) sample were examined. miRNA expression levels in CRPC were established by comparing the expression of miRNAs between CRPC and normal prostate tissue or hormone-sensitive PCa. Genome-wide gene expression studies and *in silico* analyses were performed to predict miRNA regulation in PCa and to investigate the functional significance and clinical utility of novel oncogenic pathways regulated by these miRNAs, and it was reported that miR-145-5p and miR-145-3p were downregulated in CRPC [15].

In contrast to these studies, we observed that expression of miR-145 was higher in the C4-2 PCa cells compared to normal prostate cells.

miR-200

It has been reported that all miR-200 family members are present in the serum of patients with CRPC, and high expression of these miRNAs may be prognostic in CRPC. Although the exact functions of MiR-200 are controversial, evidence clearly demonstrates the importance of the miR-200 family in PCa cell proliferation, EMT, and progression Ambs et al. [16] conducted genome-wide expression assays to evaluate miRNA profiles in 60 localized prostate tumors and 16 adjacent non-tumor prostate tissue specimens. They reported significant differences in expression of miR–32, −133, −490, −494, −520 h between these groups, as well as differences in miR–101–1/2, −200a, and −200b between patients with organ-confined PCa and those with extraprostatic disease extension.

In our study, we observed higher expression of miR-200c in PCa cells compared to the control group. Our findings are consistent with those in the literature regarding the key role of miR-200 family members in cell proliferation, EMT, and progression of PCa.

miR-125

Several studies by Shi et al. [17] have provided evidence of the role of miR-125 in PCa. In one of these studies, miR-125 expression was measured in PCa samples using *in situ* hybridization. They showed that miR-125b expression was moderately to highly elevated in PCa specimens, while androgen exposure

upregulated miR-125b in PCa cell lines. These results demonstrated that miR-125b had an oncogenic effect and contributed to PCa pathogenesis.

Fu and Cao investigated the expression levels of miR-125a-5p in PCa cell lines and human PCa biopsy specimens using RT-qPCR and reported upregulation of miR-125a-5p in the PCa cell lines [18].

Similarly, in the present study we first evaluated the level of miR-125b expression in the PCa cell line with RT-qPCR and also found that miR-125b expression level was generally upregulated in PCa cell culture.

miR-26

MiR-26a-5p is a tumor-suppressor miRNA reported to be downregulated in various tumor tissues. miR-26a-5p is a tumor suppressor miRNA that is frequently downregulated in many tumor tissues and tumor cell lines. In the study conducted by Rizzo et al., they aimed to re-express miR-26a-5p in DU-145 PCa cells and collect the genes interacting with miR-26a-5p. They further analyzed how these genes integrate with tumorigenesis-related pathways miR-26a-5p exerted an anti-proliferative effect by reducing cell survival and migration, inducing cell cycle arrest, and promoting apoptosis. Rizzo et al. [19] showed that miR-26a-5p expression reduced proliferation in DU-145 prostate tumor cells.

MiR-26a level was not significantly decreased in pre- and postoperative serum samples compared to the serum samples of controls. However, miR-26a expression level ratios were insignificantly decreased when postoperative serum samples were compared to preoperative ones [20].

Although miRNA microarray analysis has revealed a significant decrease in miR-26a in PCa tissues versus their normal counterparts, the role of miR-26a must be investigated. Zhao et al. [21] found that miR-26a expression was lower in PCa tissues compared to normal controls. Their study showed that stable miR-26a inhibited cell proliferation, metastasis, and epithelial mesenchymal transition and induced G1 phase arrest in PCa.

In the present study, there was no statistically significant difference in miR-26 expression between PCa and non-PCa cell cultures.

Let-7

Studies have shown how deregulation of some target genes can affect cellular balance in the prostate gland and promote cancer initiation, as in various human malignant neoplasms. Of the studies examining the potential effect of the let-7 miRNA family in PCa, Schubert et al. [22] reported that let-7b was a prognostic biomarker in high-risk PCa. Their findings demonstrated that let-7b has an important tumor suppressing function and the authors emphasized its potential in enhancing individualized treatment for high-risk PCa patients.

A study by Nadiminty et al. [23] showed that let-7c expression was reduced in the CRPC cell lines C4-2B, LN-IL6, and LNCaPs17 compared with controls, leading to an increase in

the synthesis of inner membrane protease-1. This resulted in increased levels of the drug efflux pump protein MDR1 in the cancer cells, which has an important role in chemoresistance. However, as let-7c also represses AR, sensitivity to androgen depletion therapy should be increased in patients with downregulated let-7c.

In our study, no significant differences in let-7c expression between the control (PNT1a) and PCa cell cultures (C4-2).

miR-32

Evidence indicates that miR-32 is an AR-modulatory miRNA that is overexpressed in CRPC and enhances PCa cell growth *in vitro*. Latonen et al. demonstrated that miR-32 increased proliferation and promoted metaplastic transformation in mouse prostate epithelium, while Ambs et al. [16, 24] reported that miR-32 was upregulated in PCa.

Jalava et al. [25] conducted a study to identify androgen-regulated miRNAs involved in the pathogenesis of CRPC and determined that 7 miRNAs were differentially expressed in benign prostatic hyperplasia (BPH) and CRPC (miR-21, miR-99a, miR-99b, miR-148a, miR-221, and miR-590-5p). They reported significantly increased growth of LNCaP cells after transfection with pre-miR-32 and pre-miR-148a, and showed that miR-32 reduced apoptosis while miR-148a increased proliferation. They also confirmed androgen regulation of miR-32 and miR-148a by expression analysis of LNCaP cells after androgen exposure. The authors concluded that androgen-regulated miR-32 was a potential marker for CRPC. We detected no significant difference in miR-32 expression level between PCa cell culture and the control group in this study.

miR-34

MiR-34a has tumor suppressing effect, inhibiting abnormal cell growth through the regulation of other genes. The expression of the miR-34 family (miR-34a, b, c) itself is regulated by transcription factor p53, which is known to play an important role in human cancer. When activated by DNA damage and oncogenic stress, p53 stimulates miR-34 transcription, resulting in induction of cell cycle arrest and apoptosis.

Hagman et al. [26] found that miR-34c expression was significantly lower in PCa specimens. In patients with higher grade disease, miR-34c levels were significantly lower than in patients with metastasis and compared to BPH samples. It has been reported that miR-34c differentiates aggressive from nonaggressive PCa and is negatively associated with PSA level. Although miR-34c expression level showed no significant correlation with response to treatment, it was significantly correlated with survival in their study. Duan et al. [27] also reported miR-34a expression was decreased in PCa tissues compared to the adjacent normal prostate tissues.

However, we detected no significant difference in miR-34 expression level between PCa cell culture and the control group in this study.

Conclusion

Many studies have investigated the diagnostic or prognostic role of miRNAs in PCa over the last decade. Although miRNAs seem inherently stable, approaches for the optimization and standardization of these molecules, are necessary to achieve high quality and reproducible results. Further research reports and analyses regarding the development of relevant standards in line with published results are still needed. Our study contributes to the literature elucidating the etiopathogenesis of PCa.

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