The Indonesian Biomedical Journal, Vol.17, No.2, April 2025, p.109-206 Print ISSN: 2085-3297, Online ISSN: 2355-9179

RESEARCH ARTICLE

miR-29 Family as Epigenetic Regulators of DNMTs in Prostate Cancer and Benign Prostatic Hyperplasia

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Received date: Nov 29, 2024; Revised date: Feb 4, 2025; Accepted date: Feb 14, 2025

Abstract

BACKGROUND: The miR-29 family (*miR-29a/b/c*) is recognized as a tumor suppressor, directly targeting DNA methyltransferases (DNMTs), key regulators of epigenetic gene silencing. Even though miR-29 has been implicated in tumor progression, its regulatory interaction with *DNMT3A/3B*, particularly in prostate cancer (PCa), has not been elucidated well. This study was conducted to explore the potential of *miR-29a/b/c* in targeting *DNMT3A/3B* in PCa and benign prostatic hyperplasia (BPH), addressing a critical gap in understanding their epigenetic role.

METHODS: This study used tissue samples that were taken surgically from 30 subjects that consisted of 15 diagnosed PCa and 15 BPH patients (as the control group), aged between 18-75 years, with urinary system disorders and had a prostate specific antigen (PSA) value between 1.18 and 56.15 ng/dL. The *miR-29a/b/c* and *DNMT3A/3B* expressions were measured using quantitative real-time PCR (qRT-PCR). The variations in mean values across groups, the associations between *miR-29a/b/c* and *DNMT3A/3B* expression levels parameters, as well as the correlation between miR-29 levels and *DNMT3A/3B* variables were then statistically analyzed.

RESULTS: The expression levels of *miR-29a/b/c* were significantly downregulated in the PCa subjects compared to the BPH subjects ($p<0.05$), and negative correlations were observed between *miR-29a/b/c* and *DNMT3A/3B* in the PCa subjects ($p<0.001$). In addition, a significant inverse correlation was detected only between *miR-29a* and *DNMT3B* in BPH subjects ($p<0.05$).

CONCLUSION: The results of this study indicated that *miR-29a/b/c* in PCa may act as a negative regulator directly targeting *DNMT3A/3B*. These findings support the role of *miR-29s* in developing miRNA-based strategies for treating PCa.

KEYWORDS: prostate cancer, benign prostatic hyperplasia, epigenetic, DNA methyltransferases-3A/3B, *miR-29a/b/c*

Indones Biomed J. 2025; 17(2): 134-42

Introduction

Cancer is a disease characterized by uncontrolled cell growth and proliferation that disrupts the normal functions of the body. Prostate cancer (PCa), one of the most common types of cancer in men, takes the second place after lung cancer.(1) Benign prostatic hyperplasia (BPH) is another common type of prostate disease in men. BPH is caused by the appearance of an abnormal number of cells (hyperplasia) that increases with age.(2,3)

According to the World Health Organization's International Agency for Research on Cancer (IARC), PCa cases differ in various regions of the world.(4) These

variations are thought to be related to the prostate specific antigen (PSA) test, which is widely used for PCa screening in developed countries. The PSA test is currently the most commonly used PCa screening method and plays a role in the diagnosis, staging and further treatment of PCa. However, the PSA level may not always be an indicator for malignant prostate disease. Although PSA test is a highly sensitive screening tool, but it is not specific in differentiating PCa from BPH.(5) Due to the limited diagnostic power of PSA tests, the search for new biomarkers for the diagnosis of PCa has been ongoing for many years. Epigenetics is potentially considered to be one of these biomarkers.

Epigenetics refers to heritable changes in gene expression that occur independently of alterations in the DNA sequence.(6) Key epigenetic mechanisms include DNA methylation, histone modifications (acetylation and methylation), and non-coding RNAs (ncRNAs).(7) DNA methylation plays a critical role in regulating gene activity during processes such as DNA repair, recombination, and replication.(8) This process involves the transfer of a methyl group from S-adenosylmethionine (SAM) to the 5'-carbon of cytosine in the CpG region, which are rich in cytosine and guanine dinucleotides. DNA methylation is mediated by DNA methyltransferases (DNMTs), which not only maintain *de novo* methylation but also act as transcriptional repressors.(9,10)

MicroRNAs (miRNAs) are small ncRNA molecules that bind to target mRNA, influencing gene expression. (11,12) Depending on their expression profiles, miRNAs can function as oncogenes or tumor suppressors.(13,14) Since the early 2000s, miRNAs have gained attention as potential biomarker in PCa due to their involvement in diagnosis, prognosis, and therapeutic responses.(11) Among the various miRNAs, the miR-29 family (miR-29a/b/c) has an important role in various pathophysiological processes reported in many studies such as targeting 677 human genes. The miR-29s has been widely observed to exhibit an inverse relationship with DNMT3A/3B across various cancers, including liver, stomach, and lung cancer. (15) In lung cancer, miR-29s have been shown to bind to the 3'UTR region of mRNA and trigger the suppression of DNMT3A/3B.(16) Research focusing on epigenetic mechanisms further indicates that the miR-29s can target DNMT3A/3B to reverse aberrant DNA methylation patterns.(17)

Despite these findings, limited studies have explored the interaction between miR-29s and DNMTs in prostate-related conditions, leaving a crucial gap in understanding their role in PCa and BPH. Investigating the expression patterns of miR-29 and its interaction with DNMTs in prostate diseases are essential for gaining deeper insights into their potential as novel biomarkers for diagnosis and prognosis. This research was also performed to provide supporting information for improving the survival outcomes in patients and the development of miRNA-based therapeutic strategies.

Methods

Subjects Recruitment and Tissue Samples Collection

In this study, 30 patients planned for prostate biopsy due to clinical suspicion of PCa who did not receive any pre-operation treatment between October 2022 and June 2023, at The Urology Polyclinic of Süleyman Demirel University Hospital, Isparta, Turkey, were included in this study. According to the histopathological evaluation of prostate core biopsies conducted by a pathologist, the subjects were categorized into two groups: 15 individuals with PCa and 15 BPH. Data on serum PSA levels, Gleason Score, and WHO 2016/ISUP (International Society of Urological Pathology) grade groups were gathered from the standard medical records of each patient. This study has been approved by the Ethical Committee of Süleyman Demirel University, Faculty of Medicine (meeting date: April 19th, 2022; the number of decisions: 132) and was conducted in accordance with the ethical guidelines of the Declaration of Helsinki.

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Detection

Total RNA of 30 fresh tissue biopsies were extracted by using a total RNA Isolation Kit (Cat. No. MG-RNA-01-100; Hibrigen, Gebze, Turkey), according to the manufacturer's protocol. The RNA quality validated by A260/A280 in NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed with TaqMan™ Advanced A.B.T.™ miR cDNA Synthesis Kit, (Cat. No. C04-01-05Atlas Biotechnology; Turkey Atlas Biotechnology, State College, PA, USA). The expressions were analyzed using the real-time quantitative polymerase chain reaction (qPCR) SYBR-Green MasterMix (Cat. No. Q03-02-01; Atlas Biotechnology) on a CFX96 real-time qPCR system (Bio-Rad, Hercules, CA, USA). The settings were configured according to the instructions provided in the kit's manual. U6 small RNA (*U6*) and *β-Actin* mRNA expression were used for normalization. The primer sequences (5'-3') were shown in Table 1.

Table 1. Specific primer sequences of miRNA and genes.

Gene	Specific Primer Sequence
U6	F: CTGCGCAAGGATGACACGCA
	R: GTCGTATCCAGTGCAGGGT
hsa-mir-29a-3p	F: AACACGCATGACTGATTTCTTTTG
	R: GTCGTATCCAGTGCAGGGT
hsa-mir-29b-3p	F: AACAAGCTTCAGGAAGCTGG
	R: GTCGTATCCAGTGCAGGGT
hsa-mir-29c-3p	F: AACAAGATCTCTTACACAGGCTGA
	R: GTCGTATCCAGTGCAGGGT
β-Actin (House Keeping)	F: CATGTACGTTGCTATCCAGGC
	R: CTCCTTAATGTCACGCACGAT
DNMT3A	F: TATTGATGAGCGCACAAGAGAGC
	R: GGGTGTTCCAGGGTAACATTGAG
DNMT3B	F: GGCAAGTTCTCCGAGGTCTCTG
	R: TGGTACATGGCTTTTCGATAGGA

The $2^{-\Delta\Delta Ct}$ comparative method was used for relative quantification of gene expression. To determine the specificity of amplification, qPCR products were evaluated using melting curves. Each sample was run in triplicate.

Statistical Analysis

Statistical analyses were performed using the SPSS 22.0 package (IBM Corporation, Armonk, NY, USA). All values are expressed as mean±standard deviation (SD). Differences in mean values between groups were analyzed using Student's unpaired t-test. Correlations between *miR-29a/b/c* expressions and *DNMT3A/3B* parameters were revealed using Pearson's correlation test. The relationship between miR-29 family levels and *DNMT3A/3B* variables was assessed by linear regression. A $p<0.05$ was statistically considered significant for all the tests.

Results

Out of 30 subjects, 15 were diagnosed with BPH and 15 with PCa, and the relevant experimental groups were formed. No difference in age was found between the groups ($p=0.679$). PSA values were found to be statistically significantly higher in the PCa group than in the BPH group ($p=0.026$). Transrectal ultrasound (TRUS)-prostate volume was found to be statistically significantly higher in the BPH group ($p=0.036$). Among the PCa subjects, most had a clinical stage of T2 which was 8 (53.3%) followed by T1 which was 7 (46.6%); and had Gleason Score of 6 which was 8 (53.3%) (Table 2).

Inverse Association between *miR-29a/b/c* and *DNMT3A/3B* Expressions in BPH and PCa Subjects

In this study, statistically significant differences was observed in the expressions of *hsa-mir-29a-3p*, *hsa-mir-29b-3p* and *hsa-mir-29c-3p* from the miR-29 family revealed by RT-qPCR analysis between PCa and BPH groups (Figure 1A-1C).

The expression of *hsa-mir-29a-3p* was upregulated in the BPH group (1.136±0.629), while it was downregulated in PCa (0.470±0.356). The *miR-29a-3p* expression was significantly decreased in PCa ($p=0.002$) (Figure 1A). The

Table 2. Clinicopathological details of patients included in the study.

Clinicopathologic Variables	BPH (n=15)	PCa (n=15)
Age (years), median (IQR)	62 (53-75)	68 (54-75)
PSA level (ng/mL), median (IQR)	3.95 (1.58-16.00)	8.60 (5.18-55.10)
TRUS-prostate volume (mL), median (IQR)	86 (40-130)	70 (45-100)
Clinical stage, n (%)		
T1	n.a.	7 (46.6)
T2	n.a.	8 (53.3)
T3	n.a.	-
Biopsy Gleason Score, n (%)		
6	n.a.	8 (53.3)
7	n.a.	2 (13.3)
8	n.a.	2 (13.3)
9	n.a.	3 (20.0)

n.a.: not applicable.

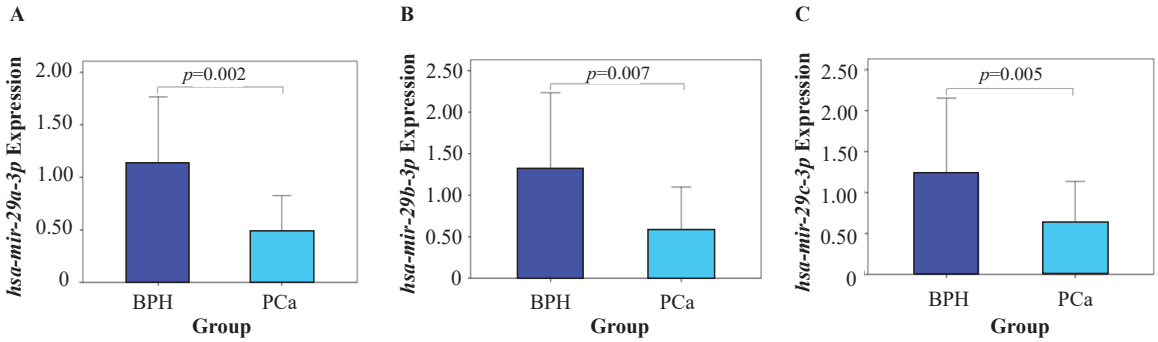

Figure 1. miR-29 family expressions of BPH and PCa groups. A: *miR-29a-3p* expression. B: *miR-29b-3p* expression. C: *miR-29c-3p* expression. *U6* was used as an internal control.

hsa-mir-29b-3p expression was downregulated in the PCa group (0.581±0.518), while it was upregulated in the BPH group (1.317±0.917). These results revealed a statistically significant decrease in *miR-29b-3p* expression in PCa (p=0.007) (Figure 1B). The mean expression of *hsa-mir-29c-3p* in PCa (0.619±0.518) and BPH (1.219±0.935) groups were statistically different (p<0.005) (Figure 1C). In summary, downregulation of the miR-29 family (*hsa-mir-29a-3p*, *hsa-mir-29b-3p* and *hsa-mir-29c-3p*) was observed in the PCa group, while the opposite was observed in BPH compared to PCa.

In this study, the expressions of *DNMT3A* and *DNMT3B* from the DNMT3 family were also analyzed by RT-qPCR method in both groups. Significant differences were observed in *DNMT3A* and *DNMT3B* gene expressions in both groups (Figure 2A-2B). The *DNMT3A* expression was significantly higher in the PCa group compared to BPH (3.929±2.156 *vs.* 1.181±0.72; (p<0.001) (Figure 2A). These results suggest that *DNMT3A* expression was significantly upregulated in the PCa group. The *DNMT3B* expression were significantly higher in the PCa group compared to BPH (5.281±2.604 *vs.* 1.382±1.142; p<0.001) (Figure 2B). *DNMT3B* expression was significantly upregulated in PCa. The results obtained from the studied DNMT3 family show that *DNMT3A/3B* mRNA expressions were significantly increased in PCa group compared to BPH in contrast to *miR-29a/b/c* expressions.

Negative Correlation between miR-29 Family and *DNMT3A/3B* Expressions in BPH and PCa

The relationship between *miR-29a/b/c* and *DNMT3A/3B* expressions were evaluated by Pearson correlation analysis. Table 3 showed the correlation coefficients and significance levels between *miR-29a/b/c* and *DNMT3A/3B* expressions in BPH and PCa tissues.

There were negative correlations between *miR-29a/b/c* and *DNMT3A/3B* expressions in both groups. In the BPH, only *miR-29a* was significantly correlated with *DNMT3B* (r=-0.675, p=0.016), while no significant correlations were found for the others. In the PCa, *miR-29a/b/c* and *DNMT3A/3B* expressions had significant negative correlations (p<0.001) (Table 3). The results of this analysis further suggest that an increasing level of the studied miR-29 family members decreases *DNMT3A/3B* expressions or a decreasing expression of the studied miR-29s leads to an enhancement of *DNMT3A/3B*. Figure 3 showed the interrelationship between *miR-29a/b/c* and *DNMT3A/3B* in BPH group that was analyzed with linear regression.

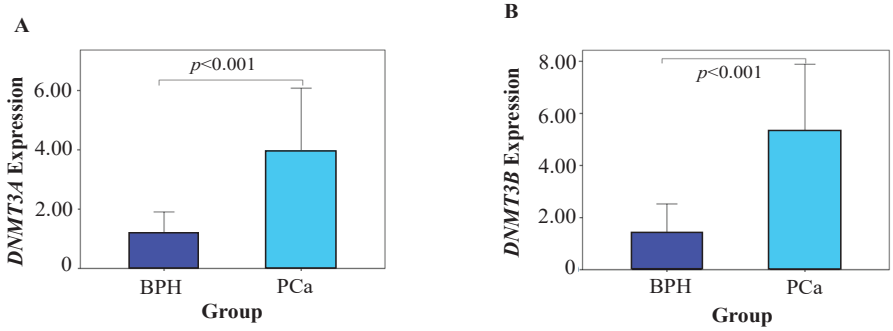

Figure 2. DNMT family expressions of BPH and PCa groups. A: *DNMT3A* expression. B: *DNMT3B* expression. *β-actin* was used as an internal control.

Table 3. Correlation coefficients and significance levels between *miR-29a/b/c* and *DNMT3A/3B* expressions in BPH and PCa tissues.

miRNA	BPH				PCa				Total			
	DNMT3A		*DNMT3B*		*DNMT3A*		*DNMT3B*		*DNMT3A*		*DNMT3B*	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
hsa-mir-29a-3p	-0.383	0.110	-0.608	0.016	-0.675	0.006	-0.873	0.000	-0.652	0.000	-0.778	0.000
hsa-mir-29b-3p	-0.101	0.500	-0.262	0.240	-0.670	0.006	-0.824	0.000	-0.544	0.002	-0.621	0.000
hsa-mir-29c-3p	-0.315	0.176	-0.174	0.372	-0.753	0.001	-0.889	0.000	-0.545	0.002	-0.539	0.002

Although a negative correlations were observed between relative *miR-29a/b/c* and *DNMT3A/3B* expression in BPH, statistically significant correlation only be found between *miR-29a* and *DNMT3B* was confirmed by linear regression analysis (R^2=0.3707, p=0.016). In general, the expression of relative miR-29 family members studied in BPH was confirmed to be negatively correlated with *DNMT3A/3B* by linear regression analysis. Increased expression of *miR-29a/b/c* resulted in decreased expression of *DNMT3A/3B* in the BPH group. On the other hand, enhanced *DNMT3A/3B* expressions were associated with decreased *miR-29a/b/c* expression. Only *miR-29a* expression was statistically significant with *DNMT3B* (p<0.05) (Figure 3B).

A linear regression analysis determined that relative *miR-29a/b/c* expressions had a statistically significant negative correlation with *DNMT3A/3B* in the PCa group in general (p<0.001) (Figure 4). In the PCa, increased *DNMT3A/3B* expression was correlated with decreased *miR-29a/b/c* expressions. The negative correlation suggests that *miR-29a/b/c* expressions contributes as a tumor suppressor.

Discussion

miR-29 is divided into two main groups: *miR-29a/miR-29b-1* on chromosome 7q32.3 and *miR-29c/miR-29b-2* on chromosome 1q32.2. The *hsa-mir-29a-3p*, *hsa-mir-*

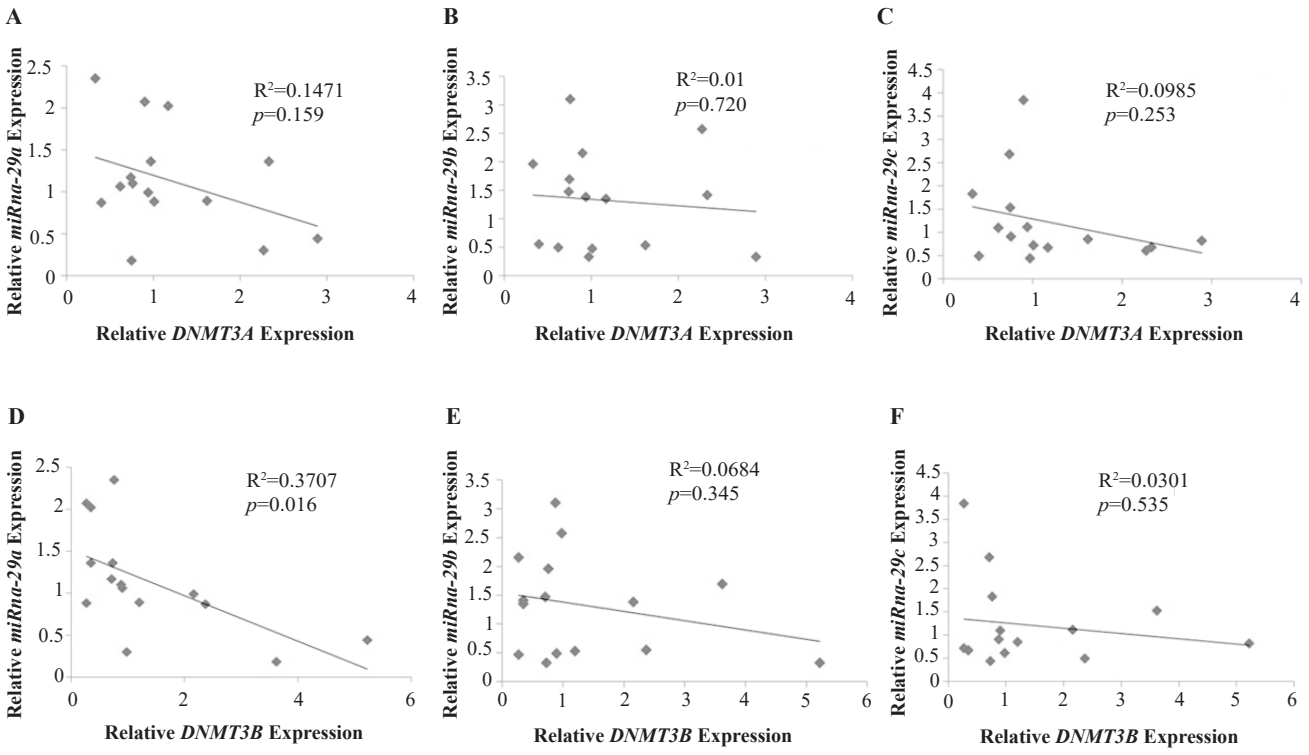

Figure 3. Linear regression analysis of the relationship between relative *miR-29a/b/c* and *DNMT3A/3B* expressions in BPH tissue.
A: Analysis between *miR-29a* with *DNMT3A*. B: Analysis between *miR-29b* with *DNMT3A*. C: Analysis between *miR-29c* with *DNMT3A*. D: Analysis between *miR-29a* with *DNMT3B*. E: Analysis between *miR-29b* with *DNMT3B*. F: Analysis between *miR-29c* with *DNMT3B*.

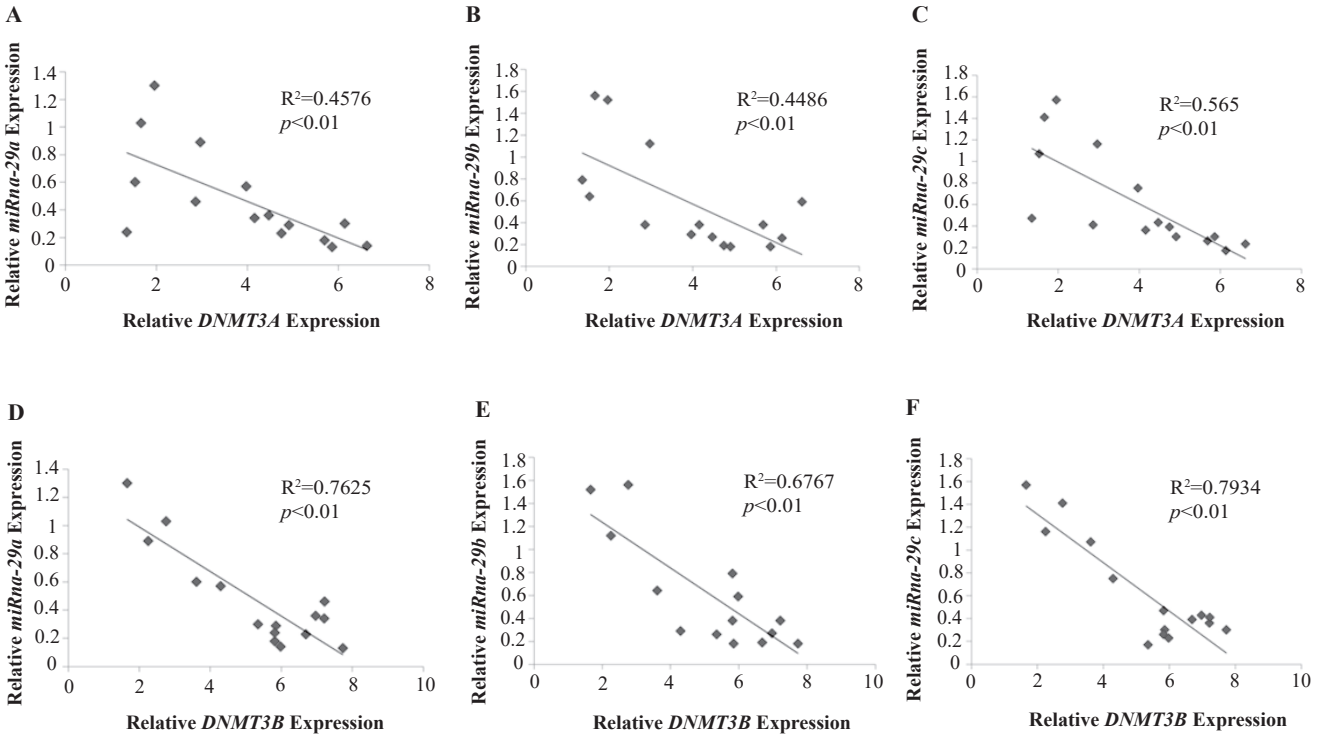

Figure 4. Linear regression analysis of the relationship between relative *miR-29a/b/c* and *DNMT3A/3B* expressions in PCa tissue. A: Analysis between *miR-29a* with *DNMT3A*. B: Analysis between *miR-29b* with *DNMT3A*. C: Analysis between *miR-29c* with *DNMT3A*. D: Analysis between *miR-29a* with *DNMT3B*. E: Analysis between *miR-29b* with *DNMT3B*. F: Analysis between *miR-29c* with *DNMT3B*.

29b-3p, and *hsa-mir-29c-3p* from the miR-29 family were evaluated in this study. Decreased miR-29 expression has been reported in various cancers, suggesting its role as a tumor suppressor and potential prognostic and diagnostic marker. It has been found downregulated in lung, gastric, bladder, ovarian, and endometrial cancers, as well as in acute lymphocytic leukemia (ALL), acute myeloid leukemia (AML), and chronic lymphocytic leukemia (CLL).(15,18) A significant downregulation of *miR-29a*, *miR-29b*, and *miR-29c* in the PCa group was also found, while their expression was elevated in the BPH compared to the PCa (Figure 1).

Significant downregulation of *miR-29a/b/c* in prostate cancer, indicating its role as a tumor suppressor.(19) miR-29 acts as a tumor suppressor in various hematological malignancies.(20) The decreased miR-29 expression with lower survival rates in mantle cell lymphoma and increased risk of metastasis and recurrence in colon cancer.(16) The increased miR-29 induces apoptosis in hepatocellular carcinoma cells, while decreased miR-29 promotes tumor growth and metastasis.(18) Studies had shown that *miR-29a* overexpression with improved survival in stage II colon cancer, while *miR-29b* downregulation was associated with increased metastasis in PCa due to its effect on the epithelial-mesenchymal transition pathway.(21-24) Additionally, upregulation of *miR-29c* in lung cancer suppressed metastasis by inhibiting cell adhesion and migration. It has been determined that each member of miR-29 can show different expression profiles in different types of cancer.(15)

Hypermethylation of tumor suppressor gene promoters can promote cancer development. The key enzymes involved are *DNMT3A* and *DNMT3B*, which are typically overexpressed in many cancers, indicating a poor prognosis.(18) This study with PCa patients showed increased *DNMT3A/3B* expressions. Another research on lung cancer reported that the DNMT3 family enhances tumor growth by silencing several tumor suppressor genes.(25) Studies have shown that the miR-29 family binds to the 3'-UTR of *DNMT3A/3B*, silencing these DNA methyltransferases. miR-29 can repress and maintain DNA methylation, targeting *DNMT3A/3B* to restore normal methylation. Tumor studies have revealed a negative correlation between *miR-29a/b/c* and *DNMT3A/3B* expression.(16,17,26) The miR-29 family negatively regulates *DNMT3A/3B* in liver, stomach, and lung cancers.(15)

Within the scope of this research, the results of studies that proved a correlation between miR-29 family and *DNMT3A/3B* expressions in BPH and PCa patient groups was also analyzed. As the result of the analysis of RT-qPCR

data, we found a statistically significant negative correlation only between *miR-29a* and *DNMT3B* in the BPH, while a non-significant negative correlation was shown with the others. On the other hand, a significant negative correlation between *miR-29a* and *DNMT3A/3B* was detected in the PCa in general.

miR-29a has a complex role in targeting, promoting demethylation of genes in leukemia but also increasing methylation by targeting Ten-Eleven Translocation (TET) and Thymine-DNA Glycosylase (TDG).(27) *miR-29a* can also decrease methylation by targeting the DNMTs in lung cancer.(28) *miR-29a* sometimes shows differential expression and regulation compared to other miR-29 members.(15)

A study of 42 BPH controls and 81 PCa patients found that *DNMT3B* gene C/T polymorphism frequencies were 26% and 20% for CC, 52% and 42% for CT, and 21% and 38% for TT, respectively. These findings suggest that *DNMT3B* polymorphism may increase promoter methylation of tumor suppressor genes, promoting prostate cancer progression.(29) Similarly, another study had shown that the C/T polymorphism in the *DNMT3B* promoter region as a risk factor for lung cancer.(30)

Research has indicated that *miR-29b* was positively associated with the progression of breast cancer.(31) Meanwhile, another study on pancreatic cancer tissue reported a negative association between *miR-29b* and *DNMT3B*, showing that *miR-29b* overexpression reduces cell viability and induces apoptosis. A luciferase reporter assay confirmed *miR-29b*'s ability to target *DNMT3B* directly. (32) *miR-29b* also negatively correlated with *DNMT3A/3B* in ovarian cancer.(33) Silencing *miR-29b* upregulates *DNMT3A/3B* expression, while *miR-29b* overexpression decreases it. It is hypothesized that *DNMT3A/3B* might also repress *miR-29b* through DNA methylation. The results of this research found a significant negative correlation between *miR-29b* and *DNMT3A/3B* in PCa patients, but a non-significant negative correlation in BPH, suggesting *miR-29b*'s effect on *DNMT3B* might be less pronounced in BPH due to other regulatory mechanisms.

In a study of 50 gastric cancer tissue samples, downregulation of *miR-29b/c* was significantly and negatively correlated with *DNMT3A* expression, which is implicated in tumorigenesis, and promoting its inhibition. Conversely, *DNMT3A* has been shown to repress *miR-29b/c* through DNA methylation processes.(21) In melanoma development, *miR-29c* expression has also been inversely correlated with *DNMT3A/3B*.(34,35) In this study, there were a significant negative correlation between *miR-29c* and *DNMT3A/3B* in the PCa, while *miR-29c* showed a non-significant negative correlation with *DNMT3A/3B* in BPH. These findings indicate that *miR-29c* may regulate *DNMT3A/3B* in PCa, however, its impact on BPH appears to be less significant compared to *miR-29a*. *miR-29c* targets the DNMT family and matrix metalloproteinase (MMP2) in pancreatic cancer tissue, where MMP2 is typically elevated. They also found that *miR-29c* expression is decreased in digestive tract cancers, and its increased expression correlates with factors such as tumor size, metastasis, and cancer stage.(34,36)

In lung cancer, an inverse correlation between miR-29 family members and *DNMT3A/3B*. Studies on non-small cell lung cancer (NSCLC) indicated that upregulation of miR-29 family expression can silence *DNMT3A/3B* mRNA, leading to demethylation of tumor suppressor genes and suppressing tumor growth. Conversely, downregulation of miR-29 family expression upregulates *DNMT3A/3B* mRNA, promoting methylation of tumor suppressor genes and facilitating tumor growth. Further investigation is needed to elucidate the various roles of *miR-29c* in the progression of BPH and its interactions with other target genes.(28) However, the miR-29 family can also act as a promoter of metastasis in certain cancers, despite its downregulation in most cases. There are reports of upregulated miR-29 members functioning oncogenically in specific cancer types.(18) For instance, upregulation of *miR-29a* in bladder cancer urine samples, with significant reduction after tumor removal.(15)

This study only includes a small sample size of 30 patients, which may restrict the generalizability of the findings, and focus solely on tissue samples from PCa and BPH patients, potentially overlooking a wider range of prostate disorders. To address these issues, future research should involve larger and more diverse cohorts, as well as longitudinal studies to better understand the temporal dynamics of miR-29 in PCa progression. Furthermore, investigating the functional mechanisms by which miR-29 regulates *DNMT3A* and *DNMT3B*, along with its interactions with other molecular pathways, could provide valuable insights for developing targeted therapeutic strategies.

Conclusion

Based on the results of this study, *miR-29a/b/c* expressions are significantly down-regulated in PCa group compared to BPH, and statistically significant negative correlation is found between miR-29s and *DNMT3A/3B* in PCa, whereas

in BPH a significant correlation was found only between *miR-29a* and *DNMT3B*. These results suggest that *miR-29a/b/c* may act as a negative regulator directly targeting *DNMT3A/3B* in prostate cancer. Therefore, the correlation between *miR-29a/b/c* and *DNMT3A/3B* might be useful in the development of novel diagnostic targets for epigenetic therapy of malignant PCa in the future.

Acknowledgments

The authors are grateful to Suleyman Demirel University, Scientific Research Fund (TYL-2022-8740) for supporting this study.

Authors Contribution

YGF and NŞC were involved in concepting and planning the research. YGF performed the experiment under the supervision of NŞ and OS. MYT and YGF carried out the laboratory work dan performed the data acquisition/collection. YGF and MC calculated the experimental data and performed the analysis. YGF drafted the manuscript and designed the figures for interpreting the results. YGF and NŞC did the proofreading, guided the preparation, and finalized the manuscript for publication. All authors took parts in giving intellectual contribution and critical revision of the manuscript.

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