European Review for Medical and Pharmacological Sciences 2021; 25: 6834-6843

Implication of miRNA-153 on PTEN expression in prostatic adenocarcinoma

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Abstract. – OBJECTIVE: A little is known about the role of miRNA-153 expression in prostate cancer (PCa), in this study we aimed to determine the prognostic value of miRNA-153 and PTEN expression in PCa, by correlating their expression with Gleason patterns, Gleason scores, and Grade groups.

PATIENTS AND METHODS: *In situ* hybridization for miRNA-153 and immunohistochemical staining for PTEN were applied on microarray sections of 80 PCa, with different Gleason grades, and 20 benign prostate hyperplasia (BPH) cases.

RESULTS: We found that miRNA-153 expression was significantly higher and PTEN was significantly lower in PCa compared to BPH. In PCa, high miRNA-153 expression and loss of PTEN expression were associated significantly with higher Gleason patterns, higher Gleason scores, and higher Grade groups. The expression of miRNA-153 showed a significant inverse correlation with PTEN expression.

CONCLUSIONS: Increased miRNA-153 expression and lost PTEN expression in PCa may provide information on their role in the progression of this cancer, suggesting that miRNA-153 could affect PTEN directly in prostatic neoplastic and hyperplastic lesions, and therefore miRNA-153 can be considered a new tool to improve the treatment efficacy and prognosis of PCa patients.

Key Words:
Immunohistochemistry, In situ hybridization, Prostate cancer, Prostate hyperplasia, MiRNA-153, PTEN.

Introduction

Worldwide, prostate cancer (PCa) is the second most frequent cancer and the fifth leading cause of cancer death in men[1]. In Egypt, PCa is the ninth most common cancer in men (2.4%) and the twelfth cause of cancer-related deaths (1.4%)[2]. Despite the low incidence of PCa in Egypt, the increase of the aging population makes this cancer to be an enormous challenge[3].

Gleason score is the most common grading system for PCa which describes the degree of morphological differentiation of the tumor[4]. This scoring system was updated to highly prognostic five Grade groups which influenced active surveillance and care management[5].

Prostate-specific antigen (PSA) is an organ-specific protein that is produced by normal, hyperplastic, and malignant cells of the prostate; however, it has a major limitation as a PCa marker due to the overlap in values between benign prostatic hyperplasia (BPH) and PCa[6].

Gleason score, Grade Group, pre-operative PSA, and clinical stage are considered pre-treatment prognostic parameters; however, they are not optimal for individual treatment decisions. PCa displays a heterogeneous set of molecular abnormalities that can explain the variable clinical outcome[7]. Therefore, there is an urgent need for reliable prognostic biomarkers that can improve early diagnosis and accurate prognosis, as well as provide targeted therapy.

MicroRNAs (miRNAs) are endogenous, single-stranded, small non-coding RNAs that are 18-25 nucleotides in length. They contribute to multiple physiological and pathological processes such as proliferation, angiogenesis, apoptosis, and tumorigenesis[8]. MiRNAs regulate gene expression via oncogenic or tumor-suppressive pathways, as well as genetic or epigenetic abnormalities such as gene amplification, rearrangement, deletion, or promoter silencing[9]. They are important post-transcriptional regulators of PTEN abundance because they may increase or decrease PTEN levels, so PTEN can act as a tu-

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mor suppressor or tumor promoter respectively[10]. Deregulation of miRNA-153 has been observed in several cancers, but until now, little is known about its clinical significance in PCa[11].

Phosphatase and tensin homolog (PTEN) is a membrane-associated phosphatase encoded by a gene on chromosome 10q23. It has an important role in many biological processes including G1 cell cycle arrest, apoptosis, cell migration-inhibition, spreading, chemotaxis, and focal adhesion formation[12]. Also, it acts as a tumor suppressor by inhibiting PI3K/AKT/mTOR, one of the most significant cell growth and pro-survival signaling pathways in cancer. PTEN loss or inactivation leads to overactivation of this signaling pathway which promotes carcinogenesis[13]. In many cancers, including PCa, PTEN is one of the most frequently deleted or mutated tumor suppressor genes.

This study aimed to assess the expression of miRNA-153 by *in situ* hybridization and PTEN by immunohistochemistry in prostatic tissue sections from Egyptian patients with PCa and BPH. Also, we compared the expression of miRNA-153 and PTEN in different Gleason patterns, Gleason scores, and Gleason Grade groups of PCa.

Patients and Methods

Samples

This study comprised 100 archival formalin-fixed paraffin-embedded blocks of prostatic specimens, divided into 80 blocks from PCa cases, all of the acinar type adenocarcinoma, and 20 blocks from BPH cases. Specimens were obtained as transurethral ultrasound biopsies (TRUS) (72 cases), transurethral resection of the prostate (TUR-P) (12 cases), and radical prostatectomy (16 cases). Blocks were collected from the Pathology Department, Theodor Bilharz Research Institute (TBRI). Blocks belonged to patients admitted to the Urology department of TBRI, and were subjected to full history, routine laboratory investigations, total and free PSA level, and imaging when indicated. All studied cases did not receive hormone therapy, chemotherapy, or radiotherapy. Patients' characteristics were summarized in Table I.

The protocol of this study was approved by the Institutional Review Board (IRB) of Theodor Bilharz Research Institute, for the protection of human subjects and adopted by the 18th world medical assembly, Helsinki, Finland (2013). The IRB waived the need for informed consent from the participants because the study was performed on stored archival tissue blocks. Personal information of these blocks' owners was anonymous and could not reasonably be used by anyone.

Tissue Microarray and Histopathological Evaluation

Tissue microarrays (TMAs) allow for high tissue production by transferring a large number of paraffin-embedded tissue cores from 'donor" blocks into "recipient" TMA blocks. At first, a morphologically representative area of interest is identified under the microscope within the preexisting donor paraffin block. Then, a hollow needle is used to remove small tissue cores (about 0.6 mm in diameter) from these regions of interest on the donor blocks to be inserted into a recipient paraffin block in a precisely spaced array pattern. Up to 20 tissue cores can be inserted in a single TMA block. Four µm sections of the prepared TMA block are cut with a microtome and are placed onto standard slides that are stained by Haematoxylin and Eosin (H&E). Sections of PCa were examined for scoring according to Gleason pattern, Gleason composite score, and Gleason Grade group[15] (Figure 1).

In Situ Hybridization (ISH)Technique of Staining

TMA Paraffin sections were melted in an oven at 60°C for 45 minutes and then stored overnight at 4°C. Sections underwent deparaffination by xylene then hydrated through degraded descending concentrations of ethanol. Proteinase-K reagent (300 µl) was applied over the tissue and incubated for 10 min at 37°C in the hybridizer, then discarded and washed twice with phosphate-buffered saline (PBS). Dehydration of slides was done through degraded ascending concentrations of ethanol. In parallel, denaturation of the probe in Exiqon ISH buffer was done by applying 25 µl hybridization mix containing the double-DIG-labeled LNA™ probe on each tissue section (HM153-100E, BIOGENEX, CA, USA), gently covered with heat-treated cover glass and sealed with Fixogum, which was removed after 24 hours using tweezers and carefully detached cover glass and placed the slides into 5× SSC. Slides were incubated with 200 µl blocking solution for 15 minutes, then in 50 µl of the anti-DIG alkaline phosphatase-conjugated antibody solution at 4°C overnight. Each slide was washed with 300 µl PBS-T and then DAB was applied. Slides were counterstained by Hematoxylin and mounted.

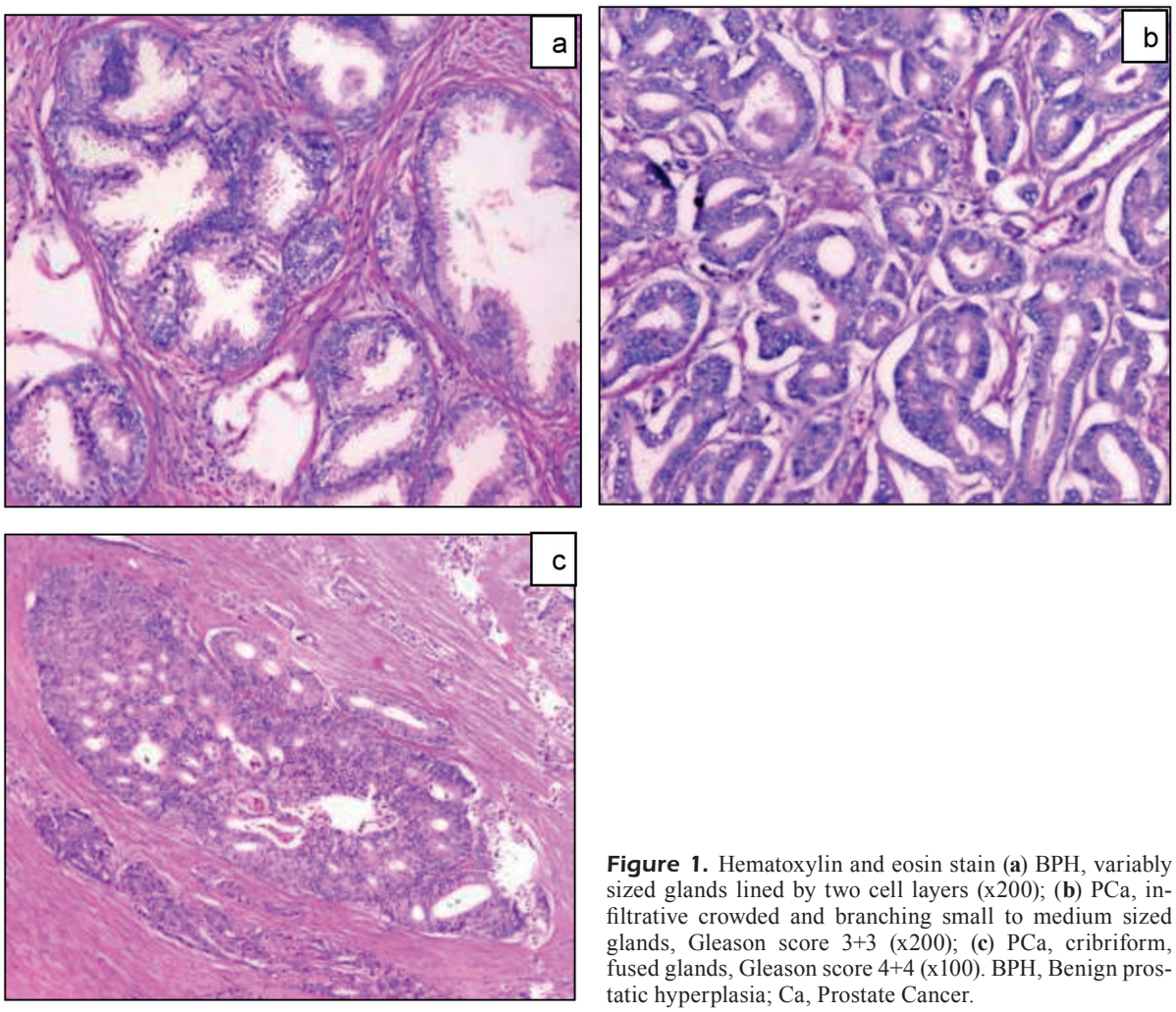

Figure 1. Hematoxylin and eosin stain (**a**) BPH, variably sized glands lined by two cell layers (x200); (**b**) PCa, infiltrative crowded and branching small to medium sized glands, Gleason score 3+3 (x200); (**c**) PCa, cribriform, fused glands, Gleason score 4+4 (x100). BPH, Benign prostatic hyperplasia; Ca, Prostate Cancer.

Immunohistochemical (IHC) Technique

TMA blocks were cut into 4 µm sections. Sections were incubated in the oven at 60°C overnight, then deparaffinized and rehydrated. Antigen retrieval was performed with 10 ml sodium citrate buffer, pH 6.0, at 90°C for 30 min. Sections were incubated in 0.03% hydrogen peroxide (EnVision/HRP, Dako) for 10 min at room temperature, to remove endogenous peroxidase activity, then were rinsed in wash buffer. Anti-PTEN monoclonal antibody (sc-393088, Santa Cruz Biotechnology; Santa Cruz, CA, USA) at dilution 1:100 was used. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Slides were rinsed in wash buffer and incubated for 30 minutes in blocking serum (0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China, and 0.5% normal goat serum X0907 (EnVision/HRP, Dako). The chromogenic reaction was carried out with 3,3′-diaminobenzidine chromogen solution for 10 minutes. Finally, after rinsing wash buffer, the slides were counterstained with hematoxylin, dehydrated in alcohol, and mounted.

Assessment of MiRNA-153 and PTEN Immunostaining

The sections were examined by using light microscope (Scope A1, Axio, Zeiss, Germany). Photomicrographs were taken using a microscope camera (AxioCam, MRc5, Zeiss, Germany). Two experienced pathologists independently examined miRNA-153 and PTEN staining while blind to the clinicopathologic data of patients. At least 10 high-power fields at 400x magnification were chosen randomly for each section.

For interpretation of miRNA-153 ISH staining, benign/malignant prostate cells with >10% nuclear staining were regarded as positive.

For interpretation of PTEN IHC staining, PTEN was considered to be negative (protein loss) if the intensity of cytoplasmic staining was markedly decreased or entirely negative across >10% of tumor cells compared to surrounding benign glands and/or stroma, which provide internal positive controls for PTEN expression[16].

Statistical Analysis

Analyses were performed using SPSS version 23 (IBM corp., Armonk, NY, USA). The significance of differences in means was calculated using One-way Anova. Pearson's Chi-square test for percentage differences. Spearman rho test was used for studying the correlation between different parameters. A p-value of < 0.05 was considered of statistical significance.

Results

Patient Characteristics

Our study included samples from 20 BPH and 60 PCa cases. The mean age for BPH cases was of 69.75 years (range, 51-83 years), whereas PCa cases had a mean age of 66.68 years (range, 49-80 years), with no significant difference between both. Serum PSA level was significantly higher in PCa compared to BPH. Also, PSA was significantly higher in Grade group 5 compared to other groups (Table I).

MiRNA153 Immunoreactivity in PCa vs. BPH

MiRNA-153 was expressed in 98.8% of PCa cases compared to 35% of BPH cases with a significant difference (p<0.001). The expression of miR-153 was significantly increased in the PCa tissues (72.38%) in comparison with the BPH (8.45%) with a significant difference (p<0.001) (Table II). Moreover, miRNA-153 expression (number of positive cases and percentage of positive cells) showed a significant increase with higher Gleason patterns (patterns 4&5), higher Gleason scores (scores 8&9), and higher Grade groups (3,4&5) of PCa compared to lower ones (Table II) (Figure 2).

PTEN Immunoreactivity in PCa vs. BPH

PTEN was only expressed in 20% of PCa cases compared to 90% of BPH cases. In other words, PTEN expression was lost in 80% of PCa cases compared to 10% in BPH. The expression

Table I. Patients' characteristics in studied prostatic lesions.

Histological diagnosis	N. of cases n. (%)	Mean Age ± SD (years)	Mean PSA ± SD (ng/ml)
Diagnosis			
BPH	20	69.75±6.78	10.68±2
PCa	80	66.68±7.66	92.87±90.25*
Gleason Pattern			
Pattern 3	27 (33.8)		
Pattern 4	36 (45)		
Pattern 5	17 (21.2)		
Composite score			
Score 6	18 (22.5)		
Score 7	14 (17.5)		
Score 8	32 (40)		
Score 9	15 (18.8)		
Score 10	1(1.25)		
Grade group			
Grade group 1	17 (21.25)	67.55±8.77	56.89±65.44
Grade group 2	7 (8.8)	65.7±7.01	66.6±41.85
Grade group 3	8 (10)	71.6±7.17	68.5±43.67
Grade group 4	31 (38.8)	64.83±6.1	96.57±94.34
Grade group 5	16 (20)	67.25±9.05	149.86±113.74**

BPH, Benign prostatic hyperplasia; PCa, Prostate Cancer; n, number; %, percentage
*p<0.001 compared to BPH.
**p<0.05 compared to the other Grade groups

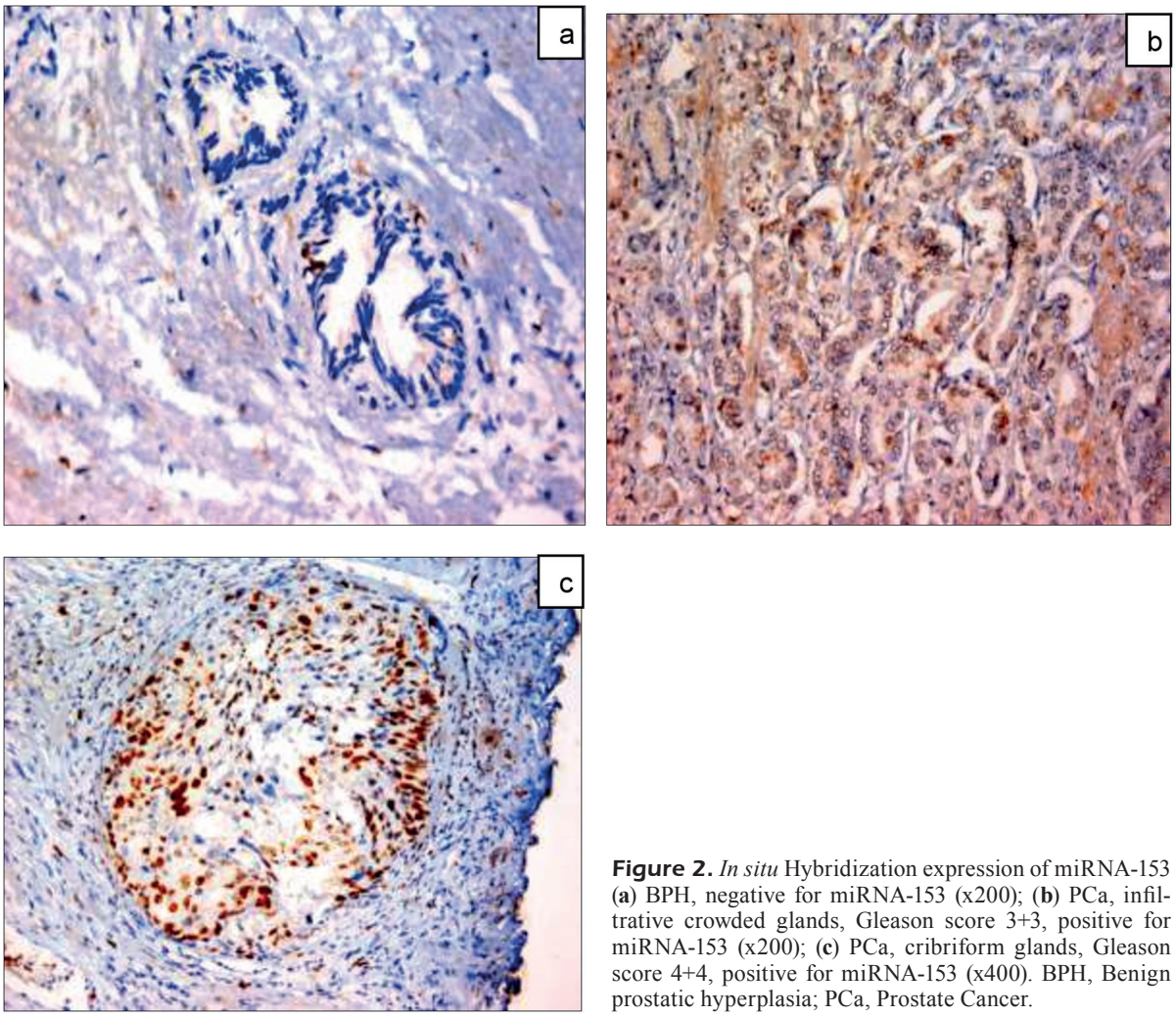

Figure 2. *In situ* Hybridization expression of miRNA-153 (**a**) BPH, negative for miRNA-153 (x200); (**b**) PCa, infiltrative crowded glands, Gleason score 3+3, positive for miRNA-153 (x200); (**c**) PCa, cribriform glands, Gleason score 4+4, positive for miRNA-153 (x400). BPH, Benign prostatic hyperplasia; PCa, Prostate Cancer.

of PTEN was significantly decreased in the PCa tissues (13.21%) compared to BPH (60%), with a significant difference (p<0.001) (Table III). Regarding PCa cases, PTEN expression (number of positive cases and percentage of positive cells) showed a significant decrease with higher Gleason patterns (patterns 4&5), higher Gleason scores (scores 8&9), and higher Grade groups (3,4&5) compared to lower ones (Table III) (Figure 3).

Correlation Between miRNA-153 and PTEN Expressions

By Spearman correlation test, serum PSA level had a significant positive correlation with miRNA-153 expression (r=0.544, p<0.001), but a significant negative correlation with PTEN expression (r=-0.537, p<0.001). In addition, there was a significant inverse correlation between miRNA-153 expression and PTEN expression (r=-0.616, p<0.001).

Discussion

Prostate cancer has the steepest age-incidence curve of all malignancies. The role of aging in causing prostate cancer is related to a myriad of changes in the genome including telomere shortening, epigenetic changes, senescence, and alterations in gene expression[17].

In our study, the mean age of BPH patients was 69.75 years, whereas it was lower in PCa (66.7 years), which is consistent with Bostwick et al[18] findings, however, the study of Hirachand et al[19] reported that PCa patients were a decade older than those with benign lesions. In anyways, pros-

Table II. *In Situ* hybridization expression of miRNA-153 in studied prostate lesions.

Item (no.)	miRNA-153 expression		
	Positive cases n. (%)	Negative cases n. (%)	Mean % of positive cells ± SD
Diagnosis			
BPH (20)	7 (35)	13(65)	8.45±5.6
PCa (80)	79 (98.8)*	1(1.2)	72.38±24.2*
Gleason Pattern			
Pattern 3 (27)	26 (96.3)	1(3.7)	53.3±22.5**
Pattern 4 (36)	36 (100)	0	85±17.6
Pattern 5 (17)	17 (100)	0	75.9±20.01
Gleason Composite Score			
Score 6 (18)	17 (94.4)	1(5.6)	53.89±24.3***
Score 7 (14)	14 (100)	0	63.67±25***
Score 8 (32)	32 (100)	0	83.75±17
Score 9 (15)	15 (100)	0	80±20
Score 10 (1)	1	0	50±0.0
Grade group			
Grade group 1 (18)	17 (94.4)	1(5.6)	53.9±24.2#
Grade group 2 (7)	7 (100)	0	50±22.4#
Grade group 3 (8)	8 (100)	0	78.75±19.6
Grade group 4 (31)	31 (100)	0	83.55±17.61
Grade group 5 (16)	16 (100)	0	78.21±21.4

BPH, Benign prostatic hyperplasia; PCa, Prostate Cancer; n, number; %, percentage
*$p<0.001$ compared to BPH.
**$p<0.001$ compared to Gleason patterns 4&5
***$p<0.001$ compared to Gleason composite scores 8&9
#$p<0.001$ compared to grade groups 3,4&5

tatic lesions were most common in the seventh decade, and both benign and malignant lesions were most common in the same age range, i.e., 60-70 years[20].

Besides the Gleason scoring and Group grading systems, the level of PSA is an important prognostic factor in PCa. No PSA level guarantees the absence of prostate cancer and the risk of cancer increases as the PSA level increases[21]. Thompson et al[22] concluded that serum PSA of 0-4 ng/ml was associated with benign lesions and value >20 ng/ml was associated with PCa.

In our study, the PSA level was significantly higher in PCa (92.87 ng/ml) than in BPH (10.68 ng/ml). This goes with Banerjee et al[23] who reported that both benign and malignant prostate lesions can cause an increase in serum PSA levels, although malignancy was more associated with rising PSA values. Furthermore, we found significantly higher PSA levels in PCa with advanced Gleason scores compared to PCa with lower scores, confirming Patel al[24] findings. On the contrary to our results, McGuire et al[25] found that high-grade, low-PSA tumors had a worse prognosis than those with higher PSA levels; this can be explained as these cancers are poorly differentiated, thus epithelial cells lose expression of a PSA encoding gene. In agreement with Koksal et al[26], we observed that PSA level was significantly negatively correlated with PTEN expression, whereas it was significantly directly correlated with miRNA-153 expression.

MiRNAs dysregulation has been reported in PCa from early to advanced stages. Several miRNAs, including miR-21, miR-22, and miR-221, have been reported to be overexpressed in PCa and can inhibit PTEN protein expression[27]. However, a greater number of miRNAs, including miRNA-100, miRNA-125, and miRNA-149 have been found to have reduced expression in PCa[9]. Only a few studies[11,27] have evaluated the role of miR-153 in PCa, and none of them employed In situ hybridization, thus little is known about its expression in the prostate.

In the current study, we investigated the expression of miRNA-153 in PCa and BPH and correlated

its association with the aggressiveness of PCa (evaluated by Gleason patterns, Gleason scores, and Grade groups). Our results revealed that 98.8% of PCa had miRNA-153 expression, which is significantly greater than the proportion of BPH (35%). Moreover, the percentage of positive cells in PCa (72.38%) increased significantly as compared to BPH (8.45%). This is in line with the results of Wu et al[27] who used the MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazoliumbromide) Assay and found upregulated miRNA-153 expression in PCa compared to adjacent noncancerous tissues, and attributed this finding to miRNA-153 role in suppressing PTEN expression and promoting PCa cell proliferation.

Our study demonstrated a significant association between miRNA-153 expression and higher Gleason patterns, Gleason scores, and Grade groups, indicating that miRNA-153 is upregulated with malignancy progression and is linked to more aggressive disease. This is consistent with the findings of Bi et al[11] who conducted quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of miR-153 on PCa and discovered that high expression of miRNA-153 was closely correlated with Gleason score and other aggressive clinical-pathological parameters. These results suggest that overexpression of miR-153 may contribute to the development of PCa. As a result, identifying this protein in PCa tissue might make it a promising therapeutic target and prognostic marker for PCa.

PTEN is a classic tumor suppressor gene that plays a key role in the homeostatic maintenance of the phosphatidylinositol-3 kinase (PI3K)/AKT cascade[28]. PTEN function is frequently lost in a

Table III. Immunohistochemical expression of PTEN in studied prostatic lesions.

	PTEN expression		
Item (no.)	Positive cases (Maintained expression) n. (%)	Negative cases (Loss of expression) n. (%)	Mean % of positive cells ± SD
Diagnosis			
BPH (20)	18 (90)	2 (10)	60±22.2
PCa (80)	16 (20)*	64 (80)	13.21±24.78*
Gleason Pattern			
Pattern 3 (27)	14 (52)**	13 (48)	32.90 ±29.6†***
Pattern 4 (36)	1 (2.8)	35 (97.2)	1.90 ±3.03
Pattern 5 (17)	1 (6)	16 (94)	5.88±24.3
Gleason Composite score			
Score 6 (18)	8 (44.4)#	10 (55.6)	34.7±34.4#
Score 7 (14)	7 (50)#	7 (50)	19.29±19.8#
Score 8 (32)	0	32 (100)	1.94±2.71
Score 9 (15)	0	15 (100)	0.00±0.00
Score 10 (1)	1 (100)	0	100±0.00
Grade group			
Grade group 1 (18)	8 (44.4)##	10 (55.6)	34.7 ± 34†
Grade group 2 (7)	6 (85.7)‡	1 (14.3)	35.7 ± 13†
Grade group 3 (8)	1 (12.5)	7 (87.5)	2.5 ± 4.62
Grade group 4 (31)	0	31 (100)	2 ± 2.73
Grade group 5 (16)	1 (6.2)	15 (93.8)	6.25±25

BPH, Benign prostatic hyperplasia; PCa, Prostate Cancer; n, number; %, percentage
*p<0.001 compared to BPH.
**p<0.05 compared to Gleason patterns 4&5
***p<0.001 compared to Gleason pattern 4&5
#p<0.001 compared to Gleason composite scores 8&9
##p<0.05 compared to Grade groups 4&5.
†p<0.001 compared to Grade groups 3,4&5.
‡p<0.05 compared to Grade groups 3,4&5

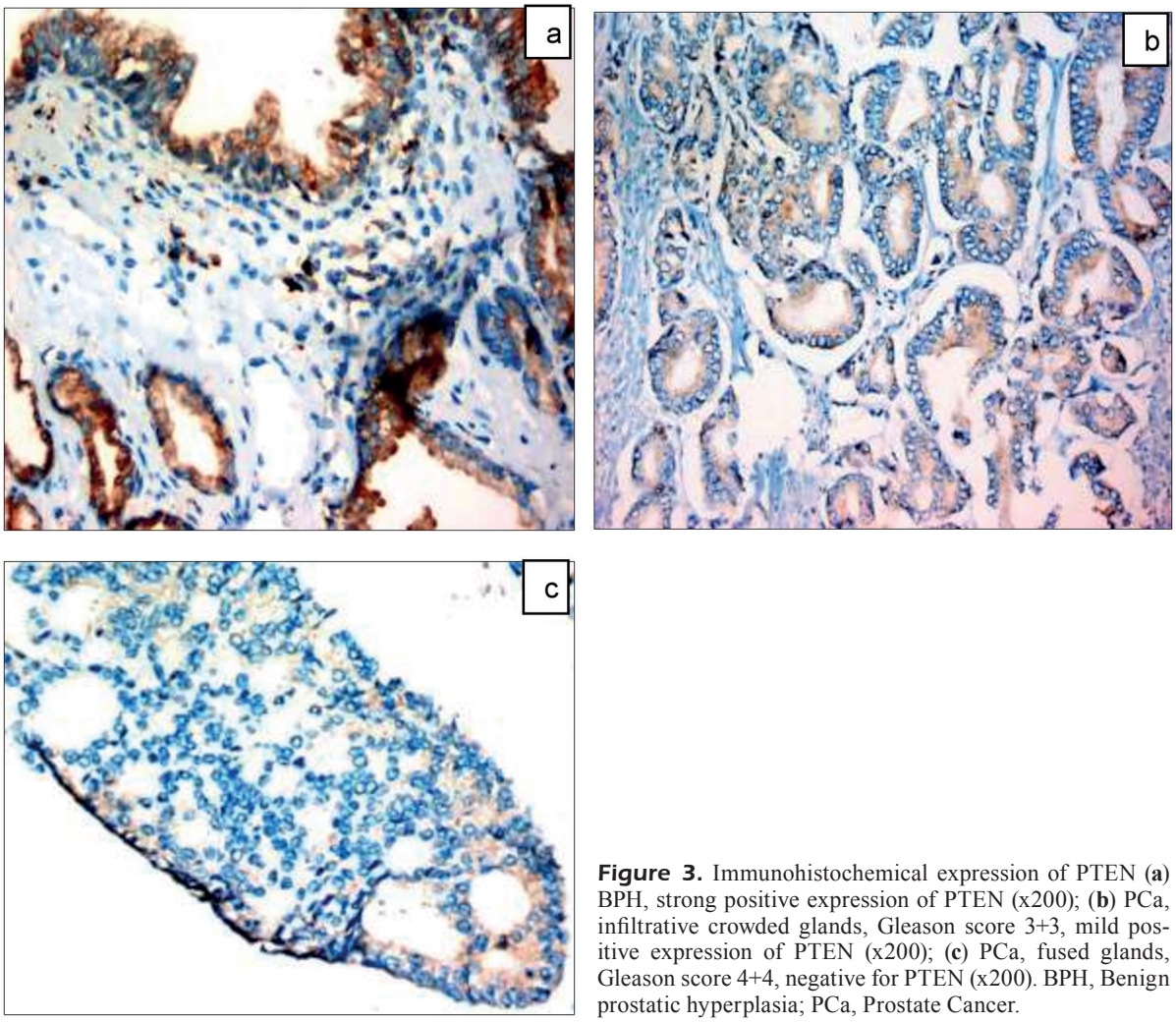

Figure 3. Immunohistochemical expression of PTEN (**a**) BPH, strong positive expression of PTEN (x200); (**b**) PCa, infiltrative crowded glands, Gleason score 3+3, mild positive expression of PTEN (x200); (**c**) PCa, fused glands, Gleason score 4+4, negative for PTEN (x200). BPH, Benign prostatic hyperplasia; PCa, Prostate Cancer.

large proportion of cancers[29]. Loss of PTEN is one of the most reliable and well-validated genetic indicators for PCa.

According to our study, PTEN was lost in 80% of PCa cases (intact in 20% of PCa), compared to a loss in 10% of BPH cases (intact in 90% of BPH). Also, it was expressed in 13.21% of cells in PCa compared to 60% of cells in BPH. These results are comparable to Al Bashir et al[30] who found PTEN loss in 59.2% of PCa cases and an intact PTEN in 98.1% of BPH cases.

Furthermore, we confirmed results of previous studies[31-33] which demonstrated an association between PTEN expression in PCa with lower Gleason patterns, Gleason scores, and Grade groups. Picanco-Albuquerque et al[34] also found that PTEN loss in Gleason score 6 (Grade Group 1) biopsies is associated with a greater probability of tumour upgrading to Gleason score 7 (Grade group 2/3) or higher in the radical prostatectomy specimens. This might indicate the usefulness of PTEN in the grading of PCa patients.

In our study, we found a significant inverse correlation between miRNA-153 and PTEN expression. This is in line with the findings of Wu et al[27] who stated that miRNA-153 suppresses PTEN expression and causes overexpression of the G1/S transitional promoter Cyclin D1 and downregulation of the cyclin-dependent kinase (CDK) inhibitor p21, promoting prostate cancer growth.

Conclusions

Loss of PTEN expression was associated with upregulation of miRNA-153 expression in PCa

and BPH tissues and vice versa. This inverse relation between miRNA-153 and PTEN suggests that miRNA-153 could influence PTEN directly in prostatic malignant and hyperplastic lesions, therefore, miRNA-153 could be a useful prognostic marker for prostate cancer and its inhibition may provide a novel targeted therapy.

Funding
The authors funded this research personally.

Conflict of Interest
The authors declare that they have no competing interests.

Authors' Contributions
All authors made a significant contribution to the conception, study design, execution, acquisition of data, analysis, and interpretation of data; took part in drafting, revising, and critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Data Availability
The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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