The Association of miR-let 7b and miR-548 with PTEN in Prostate Cancer

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Purpose: This study aims to investigate the expression level of mir-let7b-3p and mir-548, which are involved in PTEN expression in tissue samples of prostate cancer patients versus benign prostate hyperplasia (BPH) and normal adjacent tissue.

Materials and Methods: Prostate cancer tissues were obtained from patients after receiving informed consent. Total RNA extraction and cDNA synthesis were performed for determining gene expression.

Results: Ten patients were determined to have high Gleason scores (> 7), 36 and seven samples had intermediate Gleason scores (7≥) and BPH, respectively, and 40 samples were derived from normal adjacent tissue. Downregulation of mir-let7b and upregulation of mir-548 expression significantly correlated with high-risk Gleason scores.

Conclusion: The present study showed that miR-let7b and/or mir-548 can be considered as potential targets in prostate cancer therapy.

Keywords: Prostate cancer; mir-let7b; mir-548; PTEN

INTRODUCTION

One of the most frequently diagnosed malignancies in men is prostate. Various factors including environmental elements such as lifestyle, race and genetics alterations and epigenetic mechanisms influence prostate cancer and its progression toward advanced malignancy1,2. The discovery of genetic or epigenetic associations with prostate cancer in the post-genome era has improved diagnosis and management of therapy 3. PTEN tumor suppressor gene plays a key role in PI3k/AKT pathway regulation, which is the most prominent signaling pathway that regulates some cellular processes such as cell cycle, survival, metabolism, motility, genomic instability and angiogenesis, and frequent changes in prostate cancer. It was shown that the PTEN expression level decreased in 4% of primary prostate cancer and more than 40% in metastatic prostate cancer 4 . For this reason, the inhibition of PTEN suppressor can be used as a potential target therapy for metastatic prostate cancer. The early determination of prostate cancer is necessary for an effective treatment and to increase survival time. At present, the most common tools for the diagnosis of prostate cancer is serum prostate specific antigen (PSA) level 5. Sensitivity and specificity of a PSA cutoff of 4 ng/ml are about 50% and 90%, respectively. This limitation leads to low detection rate of prostate cancer in range of 4–10 ng/ml PSA level (called gray zone), and therefore, it is a disadvantage of PSA screening as a biomarker. Although PSA has decreased the mortality rate of prostate cancer, it can lead to over-diagnosis or overtreatment 6,7.

MicroRNAs (miRNAs), on average 22 nucleotides long, are a class of small noncoding RNAs and play key roles in the gene regulatory processes 8. First, they are transcribed in the nucleus by RNA polymerase II called pri-miRNA, and then they are processed into pre-miRNA by Drosha and transported to the cytoplasm to be converted to 19-25 nucleotides double strand microRNA by Dicer. MicroRNAs are transported into the RNA-induced silencing complex (RISC) that bind to 3ÚTR of the target genes and negatively regulate translation 9. The alteration of miRNA expression leads to changes in many fundamental cellular and biological processes such as differentiation, proliferation, migration, cell cycle and apoptosis that cause disease and cancer. Chromosomal rearrangement such as deletion, amplification, mutation, and methylation of promoter alter miRNA expression levels 10. Increasing miRNA expression profiling improves diagnosis, staging, progression, prognosis, and response to treatment in human cancers. Therefore, miRNAs can be used as a new oncomir or tumor suppressor mir, and new biomarkers for the diagnosis, prognosis and prediction of treatment

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Table 1. Oligonucleotide sequences were used to cDNA synthesis and amplification of miRNAs.

Oligonucleotide name	Sequence
RT6-miRlet-7b	TGTCAGGCAACCGTATTCACCGTGAGTGGTAACCAC
RT6-miR-548	TGTCAGGCAACCGTATTCACCGTGAGTGGTGCAAAA
short- miRlet-7b-rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGTGAGGTAGTAGGTTGT
short- miR-548-rev	CGTCAGATGTCCGAGTAGAGGGGGAACGGCGTCAAAACTGGCAATTAC
MP-fw	TGTCAGGCAACCGTATTCACC
MP-rev	CGTCAGATGTCCGAGTAGAGG

response. It has been demonstrated that miRNAs are very stable against heat, pH alteration, freeze-thaw and ribonuclease5. For this reason, miRNA levels are studied in different types of patient samples.

Based on information from databases/literature, which show the potential miRNAs that influence the PTEN target gene, we selected mir-let7b-3p and mir-548 for quantification by real-time polymerase chain reactions (RT-PCRs) and determined the PTEN expression levels between tumor tissues and normal tissues. In the present study, we showed dysregulation of mir-let7b and mir-548 expression levels in prostate cancer tissues.

MATERIALS & METHODS

Target prediction for microRNA

Two online computational algorithms, TargetScan (www.targetscan.org) and Diana Tools (diana.imis.athena-innovation.gr), were used for the bioinformatics prediction of miRNA binding sites.

Ethics Statement

All tests were carried out in conformity with relevant guidelines. Written informed consent was obtained from each contributor prior to tumor sample collection. All the clinical samples were obtained from Imam Khomeini Hospital of Tehran University of Medical Sciences. This study was approved by the Ethical Review Committee of Shahid Beheshti University of Medical Sciences.

Sample collection

In this study, all the samples of newly diagnosed prostate cancer cases were collected from Imam Khomeini Hospital between 2015and 2016. After informed consent, the patients for the study were selected as per the inclusion criteria: age above 40 years, high PSA, histopathological findings of the needle biopsy specimens confirmed prostate cancer or BPH, and no chemotherapy treatments were given before surgery. The exclusion criteria were: age 40 years or below, normal PSA, and frequent urination without prostate cancer symptom. Then a pathologist distinguished tumor tissue from adjacent healthy tissue as normal sample according to the pathology results of previous needle biopsy.

RNA extraction and cDNA synthesis

Total RNA extraction from the tissue samples was done with Trizol (Invitrogen Carlsbad, CA) in line with the manufacturer's protocol. The quantity of RNA samples was assessed spectrophotometrically by using Nanodrop ND-2000 (Thermo Fisher Scientific). The Mir-Q method as described by Sharbati-Tehrani11 was used for miR-let7b and mir-548 cDNA synthesis according to sequence-specific primers (**Table 1**).

Reference gene validation analysis

To validate housekeeping genes (HKGs) and to choose the most stable and reliable ones, nine of the most frequently used HKGs as internal control in RT-qPCR including SDHA, TBP, RPS13, UBC, ACTB, HSP-90AB1, PGM1, HPRT1, GAPDH named as 1, 2, 3, 4, 5, 6, 7, 8, and 9 respectively, were studied by considering their functional characteristics as well. A total number of six prostate tissue samples—three prostate carcinomas and three BPH—were included in this study. The qPCR of all the samples was carried out in triplicates, in a total volume of 20 µL containing 1X SYBR®Premix Ex Taq™II(TliRNaseH Plus) (TaKaRa), 5pM of each sense and anti-sense primer (**Table 2**), plus 1µL of cDNA template. PCR reaction was performed on the Rotor-Gene Q 5plex HRM System (Qiagene) under 30 sec enzyme activation at 95ºC, followed by 40 cycles of

Table 2. Gene symbol, primer sequence and amplicon length of selected candidate references genes

Gene symbol	Primer sequence (5′⟶3′)	Amplicon length (bp)
SDHA	F:GCAAACAGGAACCCGAGG R: CAGCTTGGTAACACATGC	202
TBP	F:TGAATAGTGAGACGAGTTCC R: TAGGGATTCCGGGAGTCAT	140
UBC	F: GCGGTGAACGCCGATGATTAT R: GATCTGCATTGTCAAGTGACG	125
HPRT1	F: CCTGGCGTCGTGATTAGTGAT R: AGACGTTCAGTCCTGTCCATTA	131
RPS13	F: AAGTACGTTTTGTGACAGGCA R: CGGTGAATCCGGCTCTCTATTAG	187
HSP90AB1	F: TCTGGGTATCGGAAAGCAAGCC R: GTGCACTTCCTCAGGCATCTTG	80
PGM1	F: AGCATTCCGTATTTCCAGCAG R: GCCAGTTGGGGTCTCATACAAA	120
ACTB	F: AGCCTCGCCTTTGCGGA R: CTGGTGCCTGGGACG	174
GAPDH	F: GAAGGTGAAGGTCGGAGTCA R: ATTGAAGGGGTCATTGATGG	109
PTEN	F: GATGATGTTTGAAACTATTCCAATG R: CTTTAGCTGGCAGACCACAA	73

Table 3. Clinical Characteristics of Patients

	Group 1	Group 2	Group 3	Group 4
Gleason Score	G > 7	G ≤ 7	BPH	Non-cancer
sample	10	36	7	40
Age(min-max)	56-70	52-81	47-83	62-85
median	64.6	65.4	65.8	71
mean	63	66.5	65	73.5
SD	5.37	6.7	14.8	8.7

95ºC for 5 sec, 58ºC for 15 sec, and 72ºC for 20 sec. We used geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004) statistical algorithms to evaluate the stability of each candidate HKG. The cycle threshold (Ct) average values of each sample's triplicates were inputted directly into BestKeeper software. For NormFinder, the average Ct of each sample was transformed to the relative quantities linear row data, using the Q = 2-Ct Equation (Livak and Schmittgen, 2001), while the normalized Ct values calculated via the Q = E (minCt-sampleCt) Equation (Q = normalized Ct value for a given gene in the current specimen, E = PCR amplification efficiency (ranging from 1 to 2 with 100% = 2), minCt = minimum Ct value for the gene among all specimens and sampleCt = the Ct value of the gene for the current specimen) were used as the input data for the geNorm program.

Quantitative Real-Time PCR

The quantitative assay of mature miRNAs was performed by SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara, Japan) by using the miR-Q method and done in the Rotor-Gene Q 5plex HRM System (Qiagene). Additionally, the expression level of PTEN was determined by using specific primers, as shown in **Table 3**. The reaction mixtures were incubated at 95°C for 30 Sec, followed by 40 cycles at 95°C for 5 Sec, and at 60°C for 30 Sec. The expression of miRNA from each sample was normalized by using the 2−ΔΔCT method relative to 5S rRNA. ΔΔCt was then computed by subtracting the ΔCt of normal tissue from the ΔCt of prostate cancer. The change in gene expression was calculated by using Equation 2−ΔΔCt 12.

Statistical analysis

Data were presented as means ± SEM and evaluated by the t-test, one way or two way analysis of variance (ANOVA) followed by the Tukey test (SPSS 24 and GraphPad prism 7.0 Software Inc., La Jolla, CA, USA). A P-value <0.05 was considered to be statistically significant. Each point or column represents the mean ± SEM (n = 4–5) ($P < 0.05$).

RESULTS

Sample collection

All the samples were divided into four groups: Gleason score >7 (10 samples), Gleason score ≤ 7 (36 samples), BPH (7 samples) and non-cancerous (40 samples). The characteristics of the patients are listed in **Table 3**.

mir-let7b and mir-548 have predictive potential binding sites in the 3'UTR of human PTEN

According to bioinformatics tools (such as DIANA tools and TargetScan) and analysis, we found that two putative binding sites of mir-let7b and mir-548 are exited in the 3'UTR of human PTEN target gene (**Figure 1**).

cDNA synthesis for miR-548 and let-7b

The mir-Q method as described by Sharbati-Tehrani

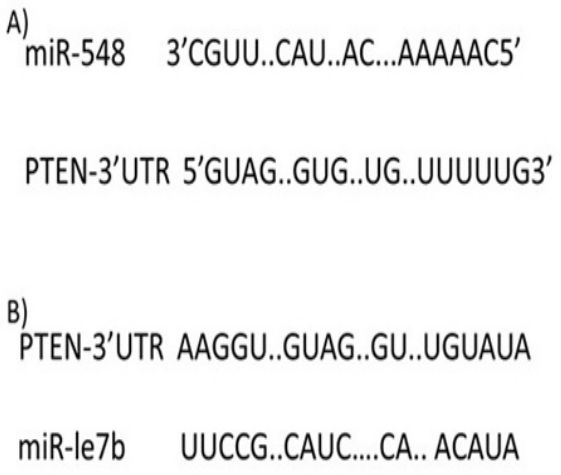

Figure 1. PTEN has two predictive banding sites for miR-548 and miR-let7b. A) Sequences of miR-548 and its putative binding sites in 3'ÚTR of PTEN. B) Sequences of miR-let7b and its putative binding sites in 3'ÚTR of PTEN.

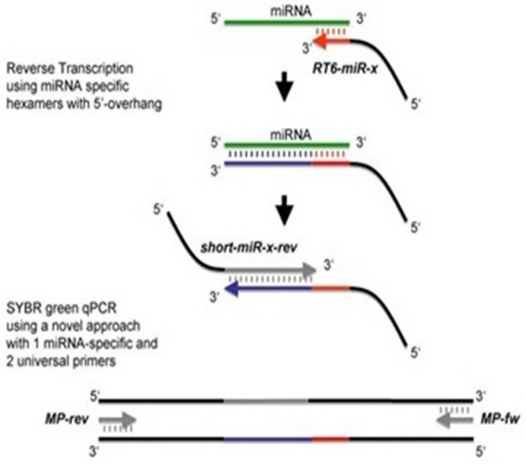

Figure 2. Schematic picture of mir-Q design. First, cDNA is synthesized by a miRNA-specific oligonucleotide that has 5' overhang (RT6-miR-x) and six complementary bases (red). Then, a single strand cDNA is converted to a double strand by a specific oligonucleotide with 5' overhang (short-miR-x-rev). Finally, amplification is performed by using two terminal universal primers (MP-fw &MP-rev) (13).

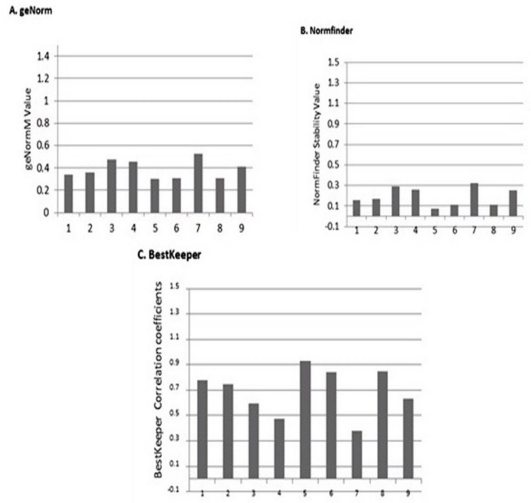

Figure 3. Candidate housekeeping genes' stability results. A) The geNorm stability results. The calculated M-value (y-axis) is plotted on the y-axis, with lower M-values corresponding to a more stably expressed gene. B) The NormFinder stability results. The calculated NormFinder stability values are plotted on the y-axis, with lower stability values corresponding to more stably expressed genes. C) The BestKeeper stability results. The calculated BestKeeper correlation coefficient is plotted on the y-axis, with higher correlation coefficient corresponding to a more stably expressed gene.

was used for miR-let7b and mir-548 cDNA synthesis according to the sequence-specific adaptor and primers (**Table 1 and Figure 2**). This method is based on the SYBR green assay.

Expression stability of candidate HKGs

Based on the M-value calculated by geNorm, all the studied HKGs revealed values lower than the cutoff value of 1.5, suggesting that all of them could be reliably used as the reference gene in the qPCR analysis of prostate tissues (**Figure 3**). The stability ranking of the nine studied HKGs from the most stable to the least stable based on geNorm M-value were 5, 6, 8, 1, 2, 9, 4, 3, and 7, respectively (**Figure 3**). The normalization factor calculated via pairwise variation values between two sequential normalization (V (n/n+1)) by taking 0.15 as a cut-off value, we suggested using two genes data for more reliable qPCR normalization (**Figure 4**). According to NormFinder stability values, the HKGs' ranking of stability from the most stable expressed to the least stable were 5, 8 4, 3, and 7, respectively (**Figure 3**). 4 and 9 also exhibited the best combination of two genes across all the samples, with a stability value of 0.003, suggesting more stability. Considering Pearson correlations, reported as the BestKeeper correlation coefficient by BestKeeper algorithm, stability ranking order of studied genes were quite in concordance with those resulted, using geNorm and NormFinder, by 5 as the most stable followed by 8, 6, 1, 2, 9, 3, 4, and 7 respectively (**Figure 3**). Finally, the expression of ACTB was used to normalize the qPCR reactions, as it was indicated to be the most stable HKG among the studied candidate HKGs by BestKeeper, geNorm, and NormFinder.

Down-regulation of let-7b expression correlated with increasing Gleason scores

Following qRT-PCR analyses, it was revealed that miR-let7b was significantly decreased in the Gleason>7 group versus the non-cancerous group. Although the expression of miR-let7b reduced in Gleason ≤7 patients, the data was not statistically significant. (*p < 0.05) (**Figure 5**).

Over-expression of mir-548 in prostate cancer tissues correlated with increasing Gleason scores

The expression levels of mir-548c in all the four groups were performed quantitatively by qRT-PCR. As shown in Figure 6, the amount of mir-548 expression level in the cancerous sample increased in high grade of prostate cancer in comparison with BPH and normal tissues as non-cancerous tissues (*P < 0.05).

DISCUSSION

The dysregulation of various miRNAs expressions are seen in prostate cancer patients, and therefore, determining the most important miRNAs and their associated pathways is very important. In this study, we decided to evaluate the expression level of mir-548c-3p in can-

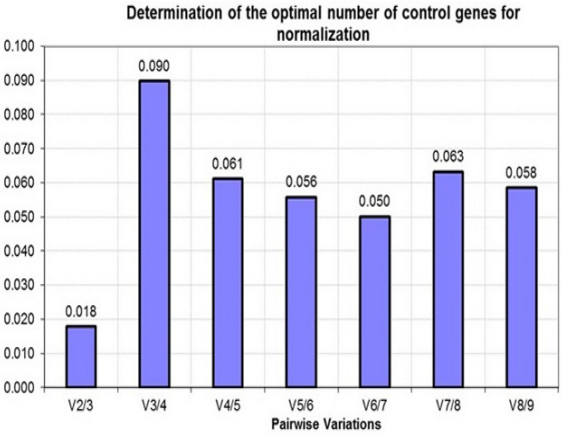

Figure 4. Determination of the optimal number of control genes required for normalization based on the pairwise variation value (Vn/n+1), which is calculated between two sequential normalization factors. The optimal number of reference genes was calculated as 2.

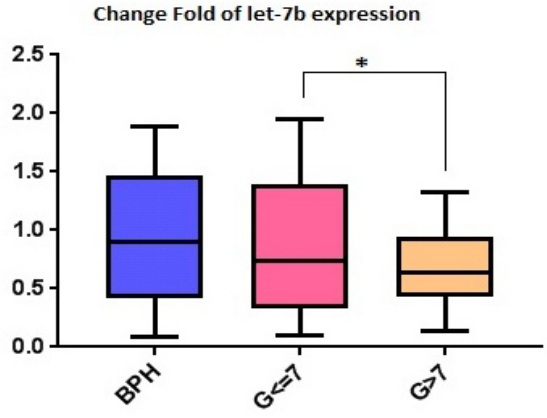

Figure 5. The qRT-PCR analysis of miR-let7b expression in prostate cancer tissues and their matched adjacent non-cancerous tissues. Data reveal that it is down regulated in both cancerous samples (Gleason scores >7 and 7≤) about more than 45% (> 45% reductions, *p < 0.05).

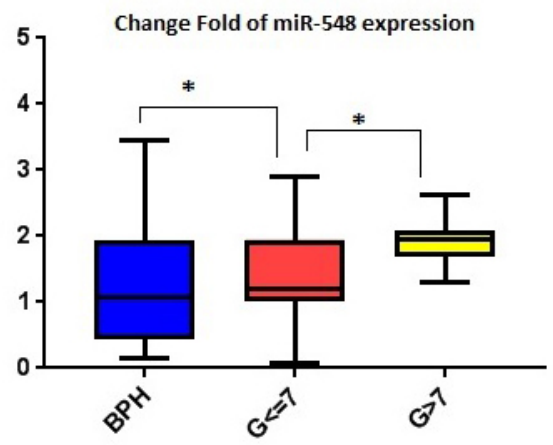

Figure 6. The expression levels of miR-548 in prostate cancer tissues and non-cancerous tissues. The qRT-PCR analysis of miR-548c expression in prostate cancer tissues (Gleason scores < 7 and Gleason scores ≥7) and their matched adjacent non-cancerous tissues. Data reveal up to more than 2-fold change in high grade prostate cancer samples compare to BPH and non-cancerous samples. The values represent the means and the error bars represent the SEM (*p < 0.05).

cer tissue samples. Previous studies have demonstrated that miRNAs play several key roles in a number of cellular pathways and biological processes such as differentiation, apoptosis, cell cycle regulation, migration and metastasis13,14.

PI3K/AKT signaling pathway has a lipid kinase family and according to their substrate and sequence homology, divided into three classes. Class-1 pertains to heterodimers and includes two subunits: catalytic and regulatory. PIP2 is a substrate of class I PI3K and converts to PIP3 as a second messenger. Then it activates downstream cascades that lead to cell growth and proliferation15.

PTEN is an antagonist of the PI3K signaling function that leads to PIP3 accumulation in cells and inhibits the activation of its downstream signals. Furthermore, PTEN is a tumor suppressor that dephosphorylates PIP3and reverses the activity of PI3K/AKT signaling pathway. Therefore, PTEN inhibits cell growth and proliferation. PTEN mutation frequencies that affect both alleles and mono allelic loss of PTEN function have been shown in different cancers such as endometrial, glioblastoma, leukemia, prostate, and breast cancers. Epigenetics phenomena including DNA methylation and microRNA decrease PTEN expression 16-18. Transcriptional silencing can describe the role of PTEN haploinsufficiency, although the loss of heterozygosity of PTEN is more seen in sporadic tumors and the severity is negatively correlated to the tumor phenotype 19. In addition, the alteration of PTEN expression can influence prognosis and response to treatment; PTEN negative tumors have shown poor response to chemotherapy drugs such as trastuzumab or cetoximab20.

Inositol polyphosphate 4-phosphatase type II (INPP4B) is another gene that acts either as a tumor suppressor or an oncogene. The negative regulation of PI3K/AKT signaling pathway by INPP4B as a tumor suppressor has been seen in various cancers, although it has been demonstrated that INPP4B expression increases in colon cancer and stimulates cell growth by the down regulation of PTEN21. In addition, overexpression of INPP4B causes the activation of PI3K/AKT pathway by the upregulation of GSK322.

As a tumor suppressor, INPP4B, similar to PTEN, converts PIP2 to PIP3, which needs activation of the PI3K/AKT signaling pathway23. In breast or prostate cancer, the loss of INPP4B function is associated with poor prognosis. Accordingly, the overexpression of INPP4B may diminish cell proliferation24. Angiogenesis, migration and invasion are inhibited by the overexpression of INPP4B in DU-145 and PC-3 prostate cancer cell lines25,26.

Cellular homeostasis needs to be balanced between protein phosphorylation and dephosphorylation. PHLPP, as a tumor suppressor, is a protein phosphatase that leads to loss of function of PHLPP, and it has been shown to be same as that of PTEN in various cancers. Hydrophobic motifs of PKC and AKT are PHLPP targets and are dephosphorylated by it. Evidence suggests that co-deletion of PHLPP and PTEN may cause metastatic progression27-29. Therefore, according to previous studies, PTEN plays a key role in the regulation of PI3K/AKT signaling pathway and the loss of PTEN function leads to malignancies.

Our study used two databases, TargetScan and Diana tools, for determining the miRNAs that have putative binding sites in 3'UTR of PTEN gene, which frequently alter in various cancers such as prostate cancer. According to this intent, miR-let7b and miR-548 were found to have two different potential binding sites in the 3'UTR of PTEN. As mentioned earlier, previous studies have shown that PTEN, as an important tumor suppressor gene, is a phosphatase family member that regulates PI3K and AKT. RNA-induced transcriptional silencing by microRNA is one the mechanisms for the down-regulation of PTEN expression. Previous studies have shown that miR-let-7b acts as a tumor suppressor mir and the expression level of miR-let-7b is downregulated in many human cancers such as prostate cancer30,31. Of course, our data showed that the miR-let7b expression level, as a tumor suppressor miRNA, is downregulated in prostate cancer, and therefore, it seems that there is no correlation between the expression levels of PTEN and miR-let7b. Additionally, we investigated the over expression of miR-548 expression level in prostate tumor tissues as compared to normal tissues adjacent.

CONCLUSIONS

This study showed that there is a difference in the expression of miR-548 in tumor and normal tissues and over expression of miR-548 is seen in prostate tumor tissues. We suggest that when miR-548c-3p increases in prostate cancer, it may repress the level of PTEN expression. Additionally, previous studies have reported that the expression levels of PTEN, INPP4B and PHLPP decrease in prostate cancer and this is why they can be used as potential targets for cancer treatment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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