miRNA-17-3/5p in prostate cancer tissues predicts clinical characteristics and is associated with PTEN

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Abstract: Prostate cancer is a common malignant tumor in men. Current diagnostic markers do not accurately reflect the progression and risk of prostate cancer. miRNA-17 may function through the degradation of the phosphatase and tensin homologue (PTEN) protein. However, the downregulation of PTEN in prostate cancer limits its application as a biomarker. Thus, we investigated the possibility that miRNA-17 may serve as a biomarker of prostate cancer. A total of 81 formalin-fixed paraffin-embedded (FFPE) specimens (35 benign tissues and 46 prostate cancer tissues) were collected from October 2013 to January 2015. PTEN was detected using immunohistochemistry, and miRNA-17-3/5p was amplified. The differential expression of PTEN/miRNA-17-3/5p between benign and malignant tissues, nonprogressive and progressive tumors, and low-risk and high-risk prostate cancer was evaluated. miRNA-17-3/5p expression levels showed negative correlations with PTEN expression. miRNA-17-3/5p expression levels exhibited gradual increases from benign to malignant tissues, nonprogressive to progressive tumors, and low-risk to high-risk prostate cancer. The sensitivity and specificity of miRNA-17-3/5p as an indicator to distinguish between benign and malignant tissues, nonprogressive and progressive tumors, and low-risk and high-risk prostate cancer were superior to those of prostate-specific antigen (PSA). Furthermore, the combination of the miRNA-17-3/5p markers showed better results than the individual markers alone. miRNA-17-3/5p may serve as an independent and stable biomarker to diagnose prostate cancer, distinguish between nonprogressive and progressive prostate cancer, and confirm the risk of prostate cancer. These results provide useful information for prognosis and treatment.

Keywords: miRNA, prostate cancer, PTEN (phosphatase and tensin homologue)

Introduction

Prostate cancer is the most common malignant tumor among males worldwide [1]. The incidence of prostate cancer in Asia has shown an increasing trend, indicating a public health issue that cannot be ignored [2-4]. The disease forms of prostate cancer includes nonprogressive disease, low-risk prostate carcinoma [5] and high-risk prostate cancer. Prostate-specific antigen (PSA) is used as a biomarker to diagnose, prognosticate and screen prostate cancer. However, this marker has clear limitations, and the diagnostic value of PSA in nonprogressive carcinoma remains controversial [6-8].

MicroRNAs are endogenous, non-coding, and short-chain RNAs (19-25 nt in length) that interact with mRNAs through a complementary base-pairing pattern to affect the production and functional expression of the downstream proteins. miRNAs have become a new tumor regulatory family with important roles in the occurrence, development, and metastasis of tumors [9, 10]. miRNA-17 plays critical roles in the evolutionary process of B-cell lymphoma and ovarian carcinoma through the deregulation of phosphatase and tensin homologue (PTEN) [11, 12]. Thus, miRNAs have significant effects on the evolutionary process of carcinoma [13].

PTEN is a reported biomarker of prostate cancer that reflects the biochemical process and predicts the tumor grade. As a tumor suppressor, loss of PTEN is closely related to prostate cancer features, including grade, stage, biochemical recurrence, metastasis and castration resistance [14, 15]. The loss of PTEN promotes the proliferation of prostate cancer cells through deregulation of the PI3K/AKT pathways [16]. However, it is difficult to observe the loss
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Table 1. Clinical demographics of patients with prostate cancer and BPH

<table>
<thead>
<tr>
<th></th>
<th>BPH</th>
<th>Prostate cancer</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>35</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>56-86</td>
<td>46-81</td>
<td>0.026</td>
</tr>
<tr>
<td>Mean</td>
<td>72.4</td>
<td>67.6</td>
<td></td>
</tr>
<tr>
<td>PSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.44-22.69</td>
<td>0-155</td>
<td>0.001</td>
</tr>
<tr>
<td>Mean</td>
<td>7.398</td>
<td>23.67</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>N/A</td>
<td>0 (0%)</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>N/A</td>
<td>29 (61.7%)</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>N/A</td>
<td>9 (19%)</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>N/A</td>
<td>9 (19%)</td>
<td>-</td>
</tr>
<tr>
<td>Gleason</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Below 6</td>
<td>14</td>
<td>6 (12.7%)</td>
<td>-</td>
</tr>
<tr>
<td>Above 7</td>
<td>11</td>
<td>41 (87.3%)</td>
<td></td>
</tr>
</tbody>
</table>

The combination of a Gleason score ≤3+3 and a tumor that did not penetrate the basement membrane was defined as non-progressive prostate cancer, whereas a Gleason score >3+3 was defined as progressive prostate cancer.

According to the classification of risk factors in prostate cancer, low-risk prostate cancer patients had a PSA <10 ng/ml, a Gleason score ≤6, or a TNM stage ≤T2a. In contrast, high-risk prostate cancer patients had a PSA >20 ng/ml, a Gleason score ≥8 or a TNM stage ≥T2c.

RNA extraction and reverse transcription

RNA was extracted from paraffin-embedded tissues of benign prostate and prostate cancer using the miRNAprep Pure FFPE Kit (TIANGEN BIOTECH (BEIJING) CO., LTD, China). Reverse transcription of the total RNA into cDNA was performed using the miRcute miRNA First-Strand cDNA Synthesis Kit (TIANGEN BIOTECH (BEIJING) CO., LTD, China).

Quantitative real-time PCR

Mature cDNA was amplified using the miRcute miRNA qPCR Detection Kit (SYBR GREEN). The qPCR primers for hsa-miR-17-3/5p were ACUGCAGUGAAGGCAUUGUAG/CAAAGUGCUU-ACAGUGCAGGUAG. Quantitative fluorescence analysis was performed using the Applied Biosystems 7500 QT-PCR machine. The PCR conditions included a holding stage at 94°C for 2 min followed by 40 cycles of 94°C for 20 s and 60°C for 34 s. All samples were normalized to the internal control U6 (small non-coding nuclear RNA). The relative expression level was analyzed using the 2^ΔΔCt method and the ΔΔCt method (ΔCt=Ct_{sample}-Ct_{U6}).

Immunohistochemistry

Prostate tissue specimens were preserved in paraffin blocks after conventional embedding in paraffin. Each specimen was used to create 2 serial sections, and the thickness of each section was 4 μm. One section was used for hematoxylin and eosin (HE) staining for diagnostic review and histological grading, and the other section was used for PTEN immunohistochemistry. The sections were conventionally deparaffinized, and antigen retrieval was performed in citric acid buffer under high tem-
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Figure 1. PTEN expression in BPH/PCA. Observation using the low power fields and high power fields showed that PTEN staining was significantly decreased. The PTEN protein expression level in benign prostate tissues was significantly higher than that in prostate cancer tissues.

The immunohistochemistry images were analyzed using the Image-Pro Plus image analysis software. First, the area of interest (AOI) with staining in the field was selected. The integrated optical density (IOD) value of this area was measured, and the area of the effective AOI was selected and measured; then, the mean optical density of this area was calculated (IOD/area, mean density). The mean value and standard deviation of the images of sections in the same experimental group were calculated. Significant differences in the mean density among all experimental groups were statistically analyzed.

Statistical analysis

Comparisons of miRNAs and the mean PTEN expression IOD between benign and malignant prostate tissues, different stages, and different risk degrees were performed using the Wilcoxon rank sum test and the Kruskal-Wallis with Dunn’s multiple comparison test. Analysis of the power of the test using two markers (miRNA-17-3/5p and PSA) to differentiate between different prostate cancer stages and risk levels was performed using logistic regression. The value of the miRNA in the diagnosis of prostate cancer was evaluated using the receiver operating characteristic (ROC) curve and the area under the curve (AUC). Curve estimation was used to analyze the correlation between the miRNA and its target protein. The statistical analyses were performed using SPSS 19.0 software. P<0.05 indicated statistical significance.
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Results

The PTEN expression level in benign prostate tissues was significantly higher than it in prostate cancer tissues. The PTEN expression level in nonprogressive prostate cancer was significantly increased compared with progressive prostate cancer tissues. The PTEN expression level in low-risk prostate cancer tissues was reduced compared with high-risk prostate cancer tissues (Figures 1 and 2).

miRNA-17-3/5p and PTEN expression levels in prostate cancer exhibited a negative correlation (Figure 3).

Differences in sensitivity and specificity when using miRNA-17-3/5p as a differential marker were determined. For differentiation between benign and malignant prostate tissues, the sensitivity and specificity of miRNA-17-3/5p were higher than those for PSA. For the differentiation between nonprogressive and progressive prostate cancer, the sensitivity and specificity of miRNA-17-3/5p were higher than those for PSA. For the differentiation between low-risk and high-risk prostate cancer, the sensitivity and specificity of miRNA-17-3/5p were higher than those for PSA (Figure 4).

Differences in sensitivity and specificity when using miRNA-17-3/5p as a differential marker were determined. For differentiation between benign and malignant prostate tissues, the sensitivity and specificity of miRNA-17-3/5p were higher than those for PSA. For the differentiation between low-risk and high-risk prostate cancer, the sensitivity and specificity of miRNA-17-3/5p were higher than those for PSA (Figure 5).

The two miRNA-17-3/5p markers were combined as a single marker to distinguish between benign and malignant prostate tissues, different stages, and different grades. The differences in sensitivity and specificity between the use of both markers together and one single marker alone were compared. For the differentiation between benign and malignant prostate tissues, the sensitivity and specificity of the com-
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**Figure 3.** The relationship between MiRNA-17-3/5p and PTEN expression in PCA. In prostate cancer tissues, the MiRNA-17-3/5p expression level showed a negative correlation with PTEN expression.
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Figure 4. MiRNA-17-3/5 expression in benign prostatic hyperplasia (BPH)/prostate cancer (PCA), nonprogressive/progressive prostate cancer and low/high risk prostate cancer. A. The MiRNA-17-3/5 expression level in prostate cancer tissues was significantly higher than that in benign prostate tissues. B. The MiRNA-17-3/5 expression level in progressive prostate cancer tissues was higher than that in nonprogressive prostate cancer tissues. C. The MiRNA-17-3/5 expression level in high risk prostate cancer tissues was higher than that in low risk prostate cancer tissues.

Combined miRNA-17-3/5p markers were increased compared with one single indicator. For the differentiation between nonprogressive and progressive prostate cancer, the sensitivity and specificity of the combined miRNA-17-3/5p markers were increased compared with one single marker. However, for the differentiation between high-risk and low-risk prostate cancer,
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Figure 5. MiRNA-17-3/5p and PSA sensitivity/specificity in the diagnosis BPH/PCA, nonprogressive/progression prostate cancer and low/high risk prostate cancer. A. The specificity and sensitivity of MiRNA-17-3/5p as individual markers to distinguish between benign and malignant prostate tissues were better than those of PSA. B. The specificity and sensitivity of MiRNA-17-3/5p as individual markers to distinguish between nonprogressive and progression prostate cancer were better than those of PSA. C. The specificity and sensitivity of MiRNA-17-3/5p as individual markers to distinguish between high risk and low risk prostate cancer were better than those of PSA.
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Figure 6. MiRNA-17-3/5p in combination and alone affect the sensitivity/specificity of the diagnosis of BPH/PCA, nonprogressive/progression prostate cancer and low/high risk prostate cancer. A. The sensitivity and specificity of MiRNA-17-3/5p as a combined marker to distinguish between benign and malignant prostate cancer tissues were better than those of the individual markers. B. The sensitivity and specificity of MiRNA-17-3/5p as a combined marker to distinguish between nonprogressive and progressive prostate cancer tissues were better than those of the individual markers. C. The sensitivity and specificity of MiRNA-17-3/5p as a combined marker to distinguish between low risk and high risk prostate cancer did not have significant differences compared to their use as individual markers.
there was no significant difference in the sensitivity and specificity of the combined miRNA-17-3/5p markers and one individual marker alone (Figure 6).

Discussion

Currently, PSA is used as a marker for the determination of benign and malignant prostate tissues and the malignant degree of prostate cancer, although deficiencies are noted. Although it is an excellent biomarker of prostate cancer, the downregulation of PTEN is difficult to observe in immunohistochemistry images. Thus, it is necessary to identify a tumor marker that can distinguish between benign and malignant tumors, between early-stage and progressive prostate cancer, and between different levels of risk. To do so, we must gain an in-depth understanding of the biological mechanisms underlying the occurrence, development, and metastasis of prostate cancer.

Recently, a large number of studies have indicated that miRNAs can regulate protein expression at the transcription level. Additionally, miRNAs can accelerate protein changes in cells from the extracellular environment or in response to environmental pressure, thereby allowing cells to accurately and effectively respond to external changes. Furthermore, cell differentiation, growth/proliferation, cell cycle control, and apoptosis are all regulated by miRNAs.

miRNAs have been shown to accurately evaluate the progression of prostate cancer and are considered independent markers for tumor prediction. Moreover, miRNA expression significantly correlates with the histological type, differentiation, invasiveness, response to chemotherapeutic drugs, and prognosis of tumors [17].

miRNA stability

As a biomarker, the stability of the miRNA is very important. Formalin fixation and paraffin embedding (FFPE) is the standard method used to preserve tissue samples for a long time and fix intracellular proteins. However, during this process, enzyme degradation and chemical degradation can damage the integrity of RNA. In general, the content of RNA extracted from FFPE is only 1/30 that of fresh tissue. Thus, only 3% of RNA can be used to reverse transcribe cDNA for quantitative RNA analysis.

Whereas RNA is unstable in FFPE samples, the miRNA content in FFPE samples is approximately the same as that in fresh tissue [18, 19], as the RNA enzyme cannot degrade the short nucleotide sequence of miRNAs. miRNAs simultaneously combine with RNA-induced silencing complex (RISC) protein, which protects them from degradation. Thus, extreme physical and chemical environments, including hypothermia, hyperthermia, strong acid, strong alkalis, formalin, and paraffin embedding, are very unstable. However, the miRNA content in 10-year-old FFPE samples is not altered compared with fresh tissue, and complete miRNAs can be extracted from only 100 ng of RNA [20-22]. The high stability of miRNAs makes them advantageous as a biomarker of prostate cancer. The miRNAs examined in our study were extracted from 3-year-old FFPE samples, and our results were relatively stable.

miRNAs have several target proteins that can reflect the pathological condition of a malignant tumor. As a standard clinical preserving method, FFPE tissue is easy to obtain and save. Furthermore, more accurate information is available regarding patient demographics for FFPE tissue, which is advantageous for retrospective research [23].

PTEN protein has phosphatase activity and can negatively regulate the PI3K/AKT signaling pathway, inhibiting the growth and proliferation of cells. Studies have showed that PTEN-deficient cell lines and tumors have high levels of activated AKT. Therefore, detection of the loss of PTEN via immunohistochemistry might suggest the presence of activated AKT. As a diagnostic marker of prostate cancer, PTEN expression is significantly decreased in advanced prostate cancer and prostate cancer patients with a poor prognosis [24, 25]. In this study, PTEN expression in benign prostate tissues was significantly increased compared with prostate cancer tissues, and the PTEN expression level gradually decreased with the malignant progression of prostate cancer.

Our results showed that miRNA-17 exhibited a negative correlation with PTEN in prostate cancer tissue, revealing its role in prostate cancer progression and its potential as a biomarker.
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[26, 27]. As a result, the sensitivity and specificity of miRNA-17 between benign and malignant as well as nonprogressive and progressive prostate cancers were increased compared with PSA.

A variety of functions for miRNA-17-3/5p have been reported in the development of prostate cancer. Some studies have also shown that miRNA-17-5p possesses oncomiR function in prostate cancer [28]. miRNA-17-5p is highly expressed in a variety of malignant tumors, including lung cancer, B cell lymphoma and breast cancer [29]. Some studies have shown that miRNA-17-3/5p functions as a tumor suppressor in the development of prostate cancer. miRNA-17-3/5p inhibits protein translation by binding to the 3′-UTR of PCAF (P300/CBP-associated factor) to inhibit the development of prostate cancer. Therefore, its expression in malignant tissues is significantly reduced compared with benign prostate tissues [28, 30].

Our study indicated that miRNA-17-3/5p expression levels were simultaneously increased in prostate cancer, which is consistent with the results of Yang et al. Their results showed that these two miRNAs had synergistic effects on the development and invasion processes of prostate cancer [26, 31]. These results indicate that miRNA-17-3/5p may promote the occurrence and development of prostate cancer with oncogene characteristics. The ability of miRNA-17-3/5p to differentiate between nonprogressive prostate cancer and progressive prostate cancer was significantly better than that of PSA; additionally, its ability to differentiate between high-risk prostate cancer and low-risk prostate cancer was also significantly better than that of PSA. These results suggest that miRNA-17-3/5p play important roles in the malignant development of prostate cancer.

Additionally, miRNA-17-5p was shown to induce tumor cells to produce rapid composite drug resistance to chemotherapeutic drugs. The detection of miRNA-17-5p in tumor cells indicates that the tumor has higher malignancy and a poorer prognosis and is prone to the development of resistance to chemotherapeutic drugs. Thus, it is better to implement early surgical treatment for prostate cancers with a high level of miRNA-17-3/5p expression because surgery has better effects than simple conservative treatment.

Therefore, we propose that miRNA-17-3/5p can be used as independent and useful markers for the diagnosis of prostate cancer, the differentiation between nonprogressive and progressive prostate cancer, and confirmation of the risk factors of prostate cancer.

Moreover, these two miRNAs not only showed high expression levels in our experiment but also exhibited synergistic effects on the regulation of the same target gene.

Some studies have further shown that these two miRNAs have different or even opposing biological functions. One miRNA demonstrated a carcinogenic function, whereas the other miRNA showed a tumor suppressor function, and selective inhibition of either could promote prostate cancer cell apoptosis [28]. Furthermore, the ratio between these two miRNAs significantly correlated with the redox reaction in cells.

Some studies on the relationship between mRNA expression and miRNA-17-92 cluster content in prostate cancer have shown that the mRNA expression level and the cluster content are independent. Additionally, the expression level of the miRNA17-92 cluster in prostate cancer cells did not correlate with androgen sensitivity [32]. Therefore, miRNA-17-3/5p have diagnostic value as members of the family and may serve as diagnostic markers of both androgen-sensitive and androgen-resistant prostate cancers.

This study showed that the expression of these two miRNAs in prostate cancer tissues was synergistic and that both had oncogene functions in prostate cancer. When these two miRNA-17-3/5p markers were combined, their ability to distinguish between benign and malignant prostate tissues and between nonprogressive and progressive prostate cancer was increased compared with one marker alone. These results indicate that these two miRNAs play important roles in the occurrence and development of prostate cancer.

Conclusion

miRNA-17-3/5p in FFEP tissue is stable upon exposure to extreme physical and chemical environments and is negatively associated with PTEN expression. Thus, miRNA-17-3/5p may serve as an independent marker for the
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diagnosis of prostate cancer, differentiation between nonprogressive and progressive prostate cancer, and clarification of the level of prostate cancer risk, which can provide useful information for the formulation of clinical treatment.

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Disclosure of conflict of interest

None.

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