

Combinations of elevated tissue miRNA-17-92 cluster expression and serum prostate-specific antigen as potential diagnostic biomarkers for prostate cancer

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Abstract. The aim of the present study was to investigate the effectiveness of the miR-17-92 cluster as a disease progression marker in prostate cancer (PCa). Reverse transcription-quantitative polymerase chain reaction analysis was used to detect the microRNA (miR)-17-92 cluster expression levels in tissues from patients with PCa or benign prostatic hyperplasia (BPH), in addition to in PCa and BPH cell lines. Spearman correlation was used for comparison and estimation of correlations between miRNA expression levels and clinicopathological characteristics such as the Gleason score and prostate-specific antigen (PSA). Receiver operating curve (ROC) analysis was performed for evaluation of specificity and sensitivity of miR-17-92 cluster expression levels for discriminating patients with PCa from patients with BPH. Kaplan-Meier analysis was plotted to investigate the predictive potential of miR-17-92 cluster for PCa biochemical recurrence. Expression of the majority of miRNAs in the miR-17-92 cluster was identified to be significantly increased in PCa tissues and cell lines. Bivariate correlation analysis indicated that the high expression of unregulated miRNAs was positively correlated with Gleason grade, but had no significant association with PSA. ROC curves demonstrated that high expression of miR-17-92 cluster predicted a higher diagnostic accuracy compared with PSA. Improved discriminating quotients were observed when combinations of unregulated miRNAs with PSA

were used. Survival analysis confirmed a high combined miRNA score of miR-17-92 cluster was associated with shorter biochemical recurrence interval. miR-17-92 cluster could be a potential diagnostic and prognostic biomarker for PCa, and the combination of the miR-17-92 cluster and serum PSA may enhance the accuracy for diagnosis of PCa.

Introduction

Prostate cancer (PCa) is the second most frequently diagnosed type of cancer in men (1). Although a number of methods for cancer therapy have been developed, PCa remains the second leading cause of cancer-associated mortality among males in economically developed countries (2). In China, the incidence of PCa has increased over the past few years, and has become the sixth most common type of cancer and the ninth leading cause of cancer-associated mortality. Although multiple molecular and signaling pathways have characterized PCa proliferation, migration, and invasion, the mechanisms remain elusive (3). Therefore, an improved understanding of the biological mechanisms underlying PCa progression may contribute to improve the clinical management and therapy. Furthermore, it is essential to improve accuracy rate for tumor detection in males, as the low specificity of prostate-specific antigen (PSA) induces unnecessary biopsies and overtreatment (4). This could be achieved by identifying other more specific biomarkers that can be useful in the diagnosis of PCa.

microRNAs (miRNAs) are a type of endogenous, small noncoding RNA. Growing evidence has demonstrated that miRNAs may serve as potential tumor suppressors and oncogenes in PCa, regulating the expression of its specific target genes involved in cell development, and metastasis (5,6). The miR-17-92 cluster, also termed oncomiR-I, is composed of six members, including miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a (7). The miR-17-92 cluster has been validated to be upregulated in several types of malignant cancer (8). Hayashita *et al* (9) suggested that overexpression of the miR-17-92 cluster may perform an important role in the development of lung cancer, particularly in small-cell lung cancer. In *Em-myc* transgenic mice, B-cell lymphomas were accelerated when the miR-17-92 cluster was transduced into hematopoietic stem cells (10). Higher expression levels

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of various miR-17-92 cluster miRNAs were significantly associated with a lower overall survival rate in patients with osteosarcoma (11). In addition, a previous study indicated that the miR-17-92 cluster was downregulated following exposure to radiation in prostate cancer LNCaP cells (12). These findings demonstrate the oncogenic role of the miR-17-92 cluster. Therefore, the present study aimed to detect the differential expression of the miR-17-92 cluster between PCa and benign prostatic hyperplasia (BPH) tissue samples from patients, as well as between PCa, and BPH cell lines. In addition, the association between miR-17-92 cluster and PCa development was investigated.

Materials and methods

Patient samples. A total of 29 PCa tissues and 16 BPH tissues were obtained from male patients who underwent surgery at Beijing Chao-Yang Hospital, Capital Medical University (Beijing, China) from January 2014 to January 2015. The mean age of patients was 68.14 years (range, 55-76 years). The experiment was conducted in accordance with the Declaration of Helsinki (World Medical Association), and the study was approved by the Ethics Committee of Beijing Chao-Yang Hospital, Capital Medical University. Written informed consent was obtained from each patient.

Cell cultures. The benign prostatic hyperplasia BPH1 cell line, and the LNCaP and PC3 PCa cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured routinely in RPMI-1640 supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 50 µg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cell lines were grown in a humidified incubator at 37°C with CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Small RNA was extracted from tissues and cell lines grown to 80% confluence using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The primers for the miR-17-92 cluster (including miR-17-3p, cat. no., CD201-0017; miR-17-5p, cat. no., CD201-0016; miR-18a, cat. no., CD201-0018; miR-19a, cat. no., CD201-0021; miR-19b, cat. no., CD201-0278; and miR-92a, cat. no., CD201-0040), miRcute miRNA first-strand cDNA synthesis kit and the miRcute miRNA qPCR detection kit (SYBR-Green; both Tiangen Biotech Co., Ltd., Beijing, China) were used to detect the miR-17-92 cluster expression according to the manufacturer's protocol. The forward primer sequence for U6 was 5'-GCA AGG ATG ACA CGC AAA TTC-3'. A universal reverse primer was provided in the miRcute miRNA qPCR Detection kit. The PCR conditions included an initiation period at 94°C for 2 min, followed by a two-step PCR program consisting of 94°C for 20 sec and 60°C for 34 sec for 40 cycles. All samples were normalized against the internal control (U6 small nuclear RNA) and analyzed using the 2^{-ΔΔC_q} method (13).

Statistical analysis. All experiments were repeated three times, and all data were analyzed using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA). Quantified data

Table I. Clinicopathological data of patients with PCa and BPH.

Characteristic	Value	
	PCa	BPH
Total, n	29	16
Mean age, years (range)	68.14 (55-76)	68.25 (49-77)
Mean PSA (range)	56.004 (0.002-1000)	5.080 (0.343-12.035)
Clinical stage, n		
T1c	2	N/A
T2c	11	N/A
T3a	6	N/A
T3b	8	N/A
T4b	2	N/A
Gleason score, n		
3 + 3	6	N/A
3 + 4	6	N/A
4 + 3	8	N/A
4 + 4	6	N/A
4 + 5	2	N/A
5 + 4	1	N/A

PSA, prostate-specific antigen; PCa, prostate cancer; BPH, benign prostate hyperplasia.

are presented as the mean ± standard deviation. Comparisons between groups were performed using one-way analysis of variance followed by all pairwise multiple comparison procedures using Bonferroni correction and Student's t-tests or nonparametric tests. In addition, Spearman's correlation was used for comparison and estimation of correlations between miRNA expression levels, and clinicopathological characteristics, including the Gleason score (14) and PSA. Receiver operating curve (ROC) analysis was performed for evaluation of specificity and sensitivity of miR-17-92 cluster expression levels for distinguishing between patients with PC, and patients with BPH. All miRNAs significantly different between the PCa and BPH group were incorporated by logistic regression, and then multiplied by the relative quantities levels for each miRNA in each patient. Biochemical recurrence-free survival times of the patients with PCa were evaluated using the Kaplan-Meier method and log-rank tests according to the cutoff value of combined miRNA score: (-4.668+0.069) x (miR-17-3p+0.023) x (miR-17-5p+0.107) x (miR-18a+0.171) x (miR-19a+0.479) x (miR-19b-0.057) x (miR-92a). P<0.05 was considered to indicate a statistically significant difference.

Results

Differential expression of miR-17-92 cluster in PCa specimens compared with BPH. Total RNA was isolated from prostate tissues of 29 males with PCa and 16 with BPH. The clinicopathological characteristics of patients are presented in

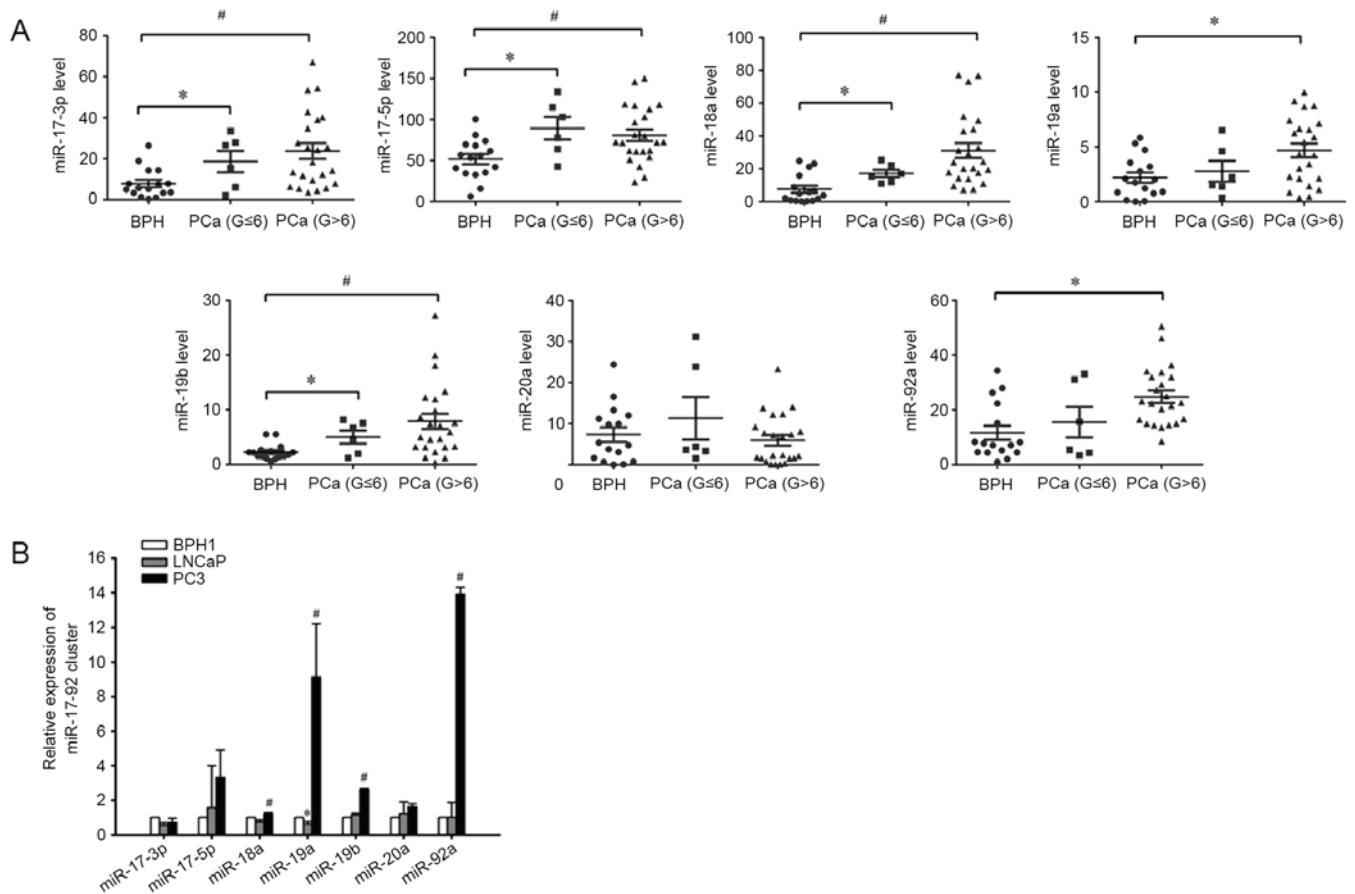


Figure 1. miR-17-92 cluster expression levels in patients with BPH, low-grade PCa, or high-grade PCa, and in BPH and PCa cell lines. (A) Expression of the miR-17-92 cluster was higher in PCa tissues compared with in BPH tissues. The expression of the miR-17-92 cluster in tissues was measured using reverse transcription-quantitative polymerase chain reaction. U6 served as an internal loading control. Each sample was detected in triplicate. *P<0.05 vs. BPH, #P<0.05 vs. BPH. (B) Expression of miR-18a, miR-19a, miR-19b and miR-92a was significantly higher in PC3 cells compared with in BPH1 and LNCaP cell lines, but the expression of miR-19a was significantly lower in LNCaP cells compared with BPH1 cells. *P<0.05 vs. BPH1, #P<0.05 vs. BPH1 and LNCaP. PCa, prostate cancer; miR, microRNA; BPH, benign prostatic hyperplasia; G, Gleason score.

Table I. The RT-qPCR results revealed significantly increased expression levels of miR-17-3p, miR-17-5p, miR-18a, miR-19a, miR-19b and miR-92a in the cancer tissues of patients with PCa compared with patients with BPH, but no significant differences in the level of miR-20a were identified between the two groups (Fig. 1A). Notably, miR-19a and miR-92a were only significantly increased in the high-grade PCa group (Gleason >6; n=23), but similar levels were maintained in patients with low-grade PCa (Gleason ≤6; n=6) and BPH (n=16) (Fig. 1A).

miR-17-92 cluster expression in prostate cell lines. The miR-17-92 cluster expression pattern was determined in cultured prostate cell lines. Three different benign and PCa cell lines were used. All of the six miRNAs in miR-17-92 cluster were detectable in the human BPH1 cell line and PCa cell lines, LNCaP and PC3. Similarly, it was revealed that the expression level of miR-18a, miR-19a, miR-19b and miR-92a were significantly increased in the human PC3 cell line compared with that in the BPH1 and LNCaP cell lines (Fig. 1B).

Correlations between miR-17-92 cluster expression and clinicopathological features in PCa specimens. Among the 29 patients with PCa, the correlations of Gleason grade

Table II. Correlation coefficients between miR-17-92 cluster expression and clinicopathological features in prostate cancer specimens.

Variable	PSA		Gleason	
	R	P-value	R	P-value
miR-17a-3p	0.018	0.928	0.602	0.001
miR-17a-5p	0.240	0.210	-0.352	0.061
miR-18a	-0.029	0.880	0.764	<0.001
miR-19a	0.121	0.532	0.468	0.011
miR-19b	-0.046	0.812	0.710	<0.001
miR-20a	-0.175	0.364	0.080	0.682
miR-92a	-0.111	0.566	0.592	0.001

PSA, prostate-specific antigen; miR, microRNA.

and PSA level with the progression risk of PCa were evaluated using bivariate correlation analysis. High expression of miR-17-3p, miR-18a, miR-19a, miR-19b and miR-92a was identified to be positively correlated with Gleason grade, but no correlation with PSA was observed (Table II).

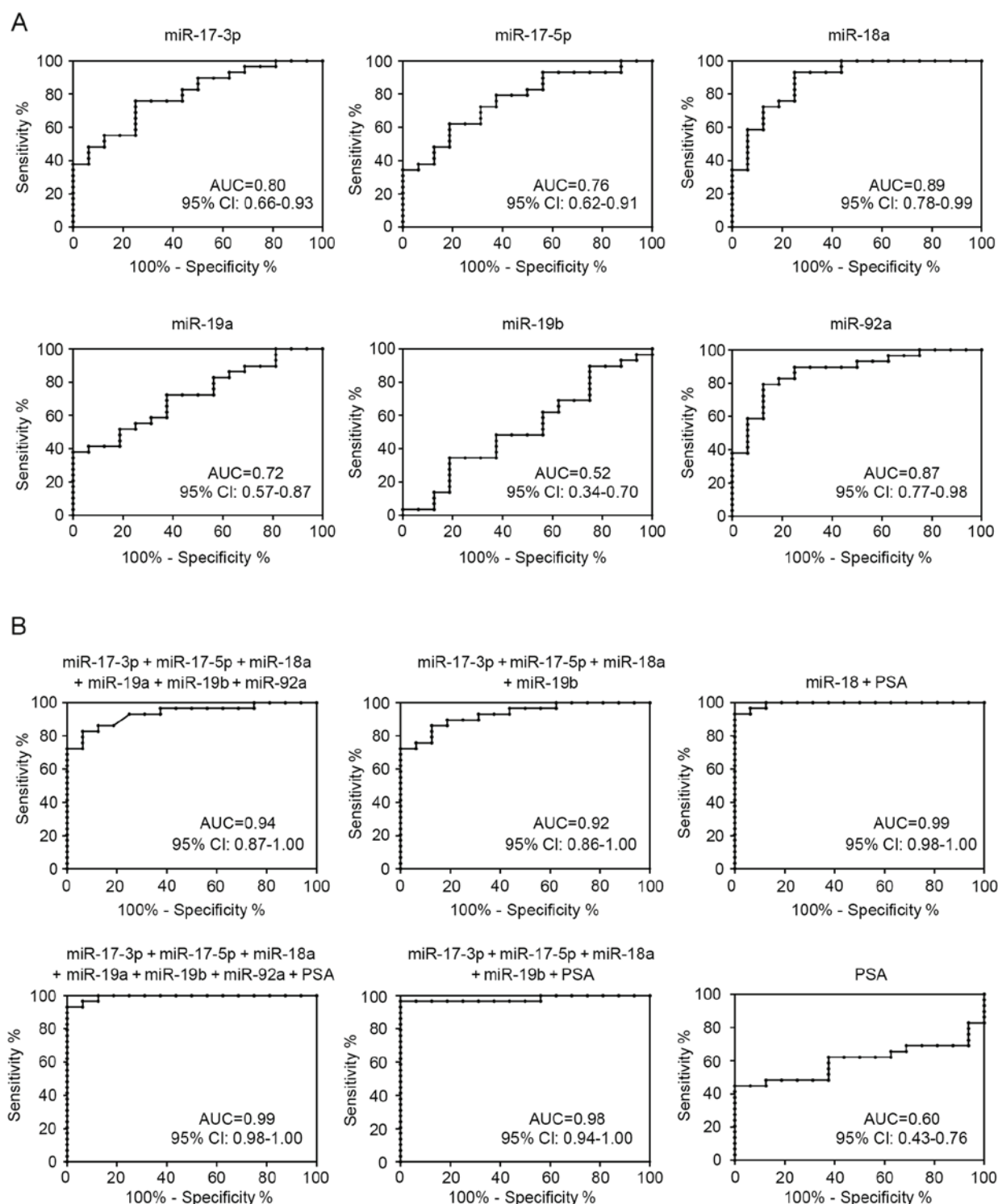


Figure 2. ROC analysis using the miR-17-92 cluster to differentiate between PCa and BPH. (A) ROCs using individual differentially-expressed miRNAs to differentiate between PCa and BPH. (B) ROCs for PSA plus individual miRNAs or a combination of all differentially-expressed miRNAs with PSA to differentiate between PCa and BPH. ROC, receiver operating curve; miR/miRNA, microRNA; PSA, prostate-specific antigen; PCa, prostate cancer; BPH, benign prostate hyperplasia; AUC, area under the curve; CI, confidence interval.

ROC analysis of the miR-17-92 cluster expression in PCa specimens. ROCs were constructed to explore the potential value of analyzed miR-17-92 cluster expression as diagnostic biomarkers for PCa (Fig. 2A). Notably, miR-18a demonstrated the most accurate discrimination [area under the ROC curve (AUC), 0.89; 95% confidence interval, 0.78-0.99; $P < 0.0001$] between PCa and BPH. At the optimal cut-off value (9.54) of

relative quantification, the sensitivity was 93.1% and specificity was 75.0%.

Combinations of miR-17-92 cluster and PSA improve diagnostic accuracy in ROC analysis. As presented in Fig. 2B, to evaluate the use of the miR-17-92 cluster as biomarkers of PCa diagnosis and progression, all the upregulated miRNAs

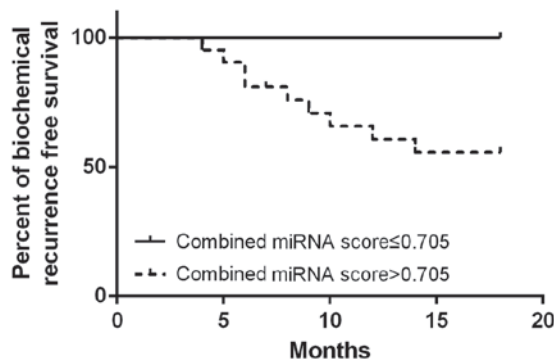


Figure 3. Survival analysis of the miR-17-92 cluster in predicting prostate cancer biochemical recurrence. miR, microRNA.

were combined to distinguish between patients with PCa and BPH. In addition, four significantly increased miRNAs in the low-grade and high-grade PCa groups, including miR-17-3p, miR-17-5p, miR-18a, and miR-19b, were also grouped together to identify the presence of PCa. These multimarker ROC analyses demonstrated higher AUC, sensitivity and specificity compared with the use of each miRNA alone (Fig. 2A). Furthermore, certain combinations of these miRNAs and PSA improved the diagnostic accuracy in ROC analysis. Considering that the miR-18a alone demonstrated the most accurate discrimination between PCa and BPH, miR-18a was combined with PSA, and the combination significantly outperformed miR-18a use alone with an AUC of 0.99.

miR-17-92 cluster is a predictor of aggressive PCa. The median follow-up time was 15.6 months. A total of 9 patients exhibited biochemical PSA recurrence. For patients with a combined miRNA score above vs. below the cutoff value (high, >0.705; n=21 vs. low, ≤0.705; n=8), a high combined miRNA score of miR-17-92 cluster was identified to be associated with shorter biochemical recurrence interval (Fig. 3).

Discussion

Accumulating studies have demonstrated that aberrant expression levels of miRNAs are involved in the development of cancer. Aberrant expression of miRNA has been identified in various tumor types, and effect essential cellular processes involved in prostate tumorigenesis, including apoptosis-avoidance, cell proliferation and migration, and the androgen signaling pathway (15). Porkka *et al* (16) identified 51 differentially-expressed miRNAs between benign and carcinoma tumors with 37 downregulated, and 14 upregulated in carcinoma samples. Lichner *et al* (17) identified 25 differentially-expressed miRNAs between the high and low biochemical failure risk groups, and revealed that specific miRNAs could aid in predicting biochemical failure risk at the time of prostatectomy.

The polycistronic miR-17-92 cluster, located in the third intron of the chromosome 13 open reading frame 25 gene, was identified by Ota *et al* (18) in 2004. Expression of these miRNAs promoted the proliferation and suppressed apoptosis of cancer cells, and was identified to be dysregulated in several types of malignant tumor (19,20). In MYC transgenic mice, lymphoma

development was accelerated following the transduction of the miR-17-92 cluster into hematopoietic cells (10). The miR-17-92 cluster was highly expressed in human hepatocellular carcinoma compared with the non-tumorous liver tissues (21). High miR-17-92 cluster expression exhibited poor prognostic implications in patients with diffuse large B-cell lymphoma (22). In addition, the miR-17-92 cluster was highly expressed in T precursor cells, and regulated lymphoproliferation in miR-17-92-transgenic models by targeting the phosphatase and tensin homolog (23). In addition, the miR-17-92 cluster was frequently and markedly overexpressed in lung cancer, particularly in those with small-cell lung cancer (9,24). The upregulation of the miR-17-92 cluster serves an important role in breast tumorigenesis and cell invasion, which contributes to the response of triple-negative breast to caloric restriction used in combination with radiation (25). A previous study reported that the miR-17-92 cluster was implicated in cervical cancer progression (26). Altogether, these findings demonstrated the potential of miR-17-92 cluster to disrupt the homeostasis of multiple organs and induce malignancy.

miRNAs in the miR-17-92 cluster have been demonstrated to function as oncogenes in PCa cells. Sylvestre *et al* (27) suggested that miRNAs from the miR-17-92 cluster serves as oncogenic miRNAs when overexpressed, by acting on essential regulators of the cell cycle and apoptosis. In addition, the miR-17-92 cluster can harbor androgen receptor binding sites, and their expression is sensitive to androgen stimulation in DUCaP and LNCaP cells (28). Members of the miRNA-17-92 cluster were also identified in PCa progression. Yang *et al* (29) confirmed that miR-17-5p and miR-17-3p could enhance PCa growth, and invasion by repressing the same target tissue inhibitor of metalloproteinase 3. Furthermore, serum levels of miR-19a and miR-19b were upregulated in patients with PCa (30). In contrast to the present results, the expression level of miR-20a was significantly higher in patients with a Gleason score of 7-10 compared with the patients with a Gleason score of 0-6, supporting the oncogenic role of miR-20a in PCa (31). This discrepancy may have been caused by differences in the source of miRNA obtained and the detection methods used.

Of note, miR-18a is a highly expressed miRNA in several types of cancer. The concentrations of miR-18a in plasma/serum of patients with cancer, including esophageal, pancreatic, hepatocellular and colorectal cancers, were all higher compared with that of healthy volunteers (32). Shen *et al* (33) revealed that the expression levels of miR-18a increased in lung cancer tissues, and Su *et al* (34) demonstrated that miR-18a could be a promising biomarker for the detection of gastric cancer, and its upregulation may be associated with prognosis of bladder cancer. Similar to these results, the present study also identified miR-18a to be upregulated in patients with PCa, and ROC analysis demonstrated that the increase of miR-18a in PCa tissues was the most accurate diagnostic biomarker and could distinguish patients with PCa from controls with high sensitivity and specificity.

Several studies have reported that numerous miRNAs may be independent biochemical recurrence prediction markers (35-38). The present study aimed to investigate the potential of miR-17-92 cluster derived from surgery at the initial visit in predicting the possibility of PCa progression or metastasis, and the results obtained demonstrated that

the expression status of the miR-17-92 cluster was a good prognostic marker for time to progression to biochemical recurrence.

To the best of our knowledge, the present study was the first to demonstrate that the miR-17-92 cluster was upregulated in PCa tissue samples as compared with BPH control tissue, which suggests that these miRNAs are contributors to PCa oncogenesis. Furthermore, the high expression of the miR-17-92 cluster demonstrated increased sensitivity and specificity compared with PSA on diagnosis of PCa. Finally, the present findings suggested the high expression of the miR-17-92 cluster could be an independent prognostic marker for PCa progression. Therefore, miR-17-92 cluster is a potential diagnostic and prognostic biomarker for PCa, and possesses clinical utility to identify between patients with PCa and BPH. A combination of the miR-17-92 cluster and serum PSA can enhance the accuracy for diagnosis of PCa. However, the cellular mechanisms for the changes in miRNA levels in the prostate tissues with pathological progression are yet to be elucidated.

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