

MicroRNA expression profiles in benign prostatic hyperplasia

NAN ZHANG^{1*}, ZHONGYI LI^{1*}, FUDING BAI¹, NA JI²,
YICHUN ZHENG¹, YI LI¹, JIMIN CHEN¹ and XIAWA MAO¹

Departments of ¹Urology and ²Anesthesia, The Second Affiliated Hospital,
Zhejiang University School of Medicine, Hangzhou, Zhejiang 310009, P.R. China

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Abstract. Although alterations in microRNA (miRNA) expression have been previously investigated prostate cancer, the expression of miRNAs specifically in benign prostate hyperplasia (BPH) of the prostatic stroma remains to be fully elucidated. In the present study, miRNAs and gene expression profiles were investigated using microarray analysis and reverse transcription quantitative-polymerase chain reaction (RT-qPCR) in BPH tissue to clarify the associations between miRNA expression and target genes. Prostate tissue samples from five patients with BPH and five healthy men were analyzed using human Affymetrix miRNA and mRNA microarrays and differentially expressed miRNAs were validated using RT-qPCR with 30 BPH and 5 healthy control samples. A total of 8 miRNAs, including miRNA (miR)-96-5p, miR-1271-5p, miR-21-3p, miR-96-5p, miR-181a-5p, miR-143-3p, miR-4428 and miR-106a-5p were upregulated and 8 miRNAs (miR-16-5p, miR-19b-5p, miR-940, miR-25, miR-486-3p, miR-30a-3p, let-7c and miR-191) were downregulated. Additionally, miR-96-5p was demonstrated to have an inhibitory effect on the mRNA expression levels of the following genes: Mechanistic target of rapamycin (MTOR), RPTOR independent companion of MTOR complex 2, syntaxin 10, autophagy-related protein 9A, zinc finger E-box binding homeobox 1, caspase 2 and protein kinase c ϵ . Additionally, 16 differentially expressed miRNAs were identified using RT-qPCR analysis. This preliminary study provides a solid basis for a further functional study to investigate the underlying regulatory mechanisms of BPH.

Introduction

Benign prostate hyperplasia (BPH) is one of the most common diseases in the aging male population that seriously affects patients' quality of life (1). It is estimated that BPH occurs in 30-60% of men \geq 50 years in mainland China, with the disease incidence increasing with age (2). In an aging population, this disease is emerging as an important clinical topic. Although a wide variety of drugs may be used to treat BPH, there are limitations in the application of these drugs due to drug dependence and severe side effects (3,4). A previous study demonstrated that microRNA (miRNA) dysregulation is involved in the development and progression of BPH (5). However, the exact involvement of miRNAs in BPH is not fully understood, due to the complex interaction between miRNAs and gene expression levels.

miRNAs are single-stranded non-coding RNAs of ~22 nucleotides (6) that control gene expression at the post-transcriptional level by mRNA cleavage or translational suppression (6). Currently, there are 2,603 human mature miRNAs registered at miRNA database miRBase release 21.0, and the number of newly discovered miRNAs is gradually increasing. Previous studies demonstrated that miRNAs have key regulatory roles in numerous biological processes, including development, differentiation, signal transduction and cell maintenance (7,8). miRNA dysregulation is also a common feature of various diseases, including cancer, inflammation, autoimmune disease and prostate hyperplasia (9,10).

To date, despite several studies reporting that miRNA mediates prostate cancer, few articles have investigated miRNA regulation in BPH (11-13). To the best of our knowledge, only two studies have examined miRNA expression in BPH and healthy controls, which provided inconsistent findings (14,15). Furthermore, limited microarray data has been confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and only one previous study has reported an association between *Homo sapiens* (hsa)-miR-143 or hsa-miR-145 and clinicopathological data in BPH (14). In the present study, miRNA and gene expression profiles in prostate tissue of patients with BPH and healthy men were investigated using microarray analysis and RT-qPCR.

Materials and methods

Patients and tissue samples. Prostate tissue samples were collected from 5 patients with BPH and 5 healthy volunteers

Correspondence to: Dr Xiawa Mao or Dr Nan Zhang, Department of Urology, The Second Affiliated Hospital, Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou, Zhejiang 310009, P.R. China

E-mail: mao_xiawa@zju.edu.cn

E-mail: nanzhang@zju.edu.cn

*Contributed equally

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(aged 42±10 years) at The Second Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China) between December 2016 and January 2017. The participants included in the current study had not received any medical treatment prior to tissue collection. A total of 10 prostate tissue samples were immediately snap-frozen in liquid nitrogen following radical prostatectomy. Extracted RNA was used for microarray analyses and RT-qPCR. An additional 25 BPH samples (aged 43±8 years) were included in the RT-qPCR assay between January 2017 and April 2017 at The Second Affiliated Hospital, Zhejiang University School of Medicine. Clinicopathological information for each patient was obtained, including age and levels of preoperative prostate-specific antigen. The present study was conducted with the approval of The Ethics Review Board at Zhejiang University School of Medicine (Hangzhou, China) and all participants provided written informed consent.

Cell culture. WPMY-1 cells obtained from the cell bank at the Chinese Academy of Sciences (Shanghai, China) were cultured in DMEM medium (11965-092; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 100 U/ml penicillin/streptomycin and 10% fetal bovine serum (15140-122 and 10099-141 both from Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂, as previously described (16).

RNA extraction. RNA was extracted with the miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. A NanoDrop ND-1000 spectrometer (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) was used to detect the RNA yield and 260/280 nm ratio. A 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to quantify the RNA integrity number.

miRNA and mRNA microarray experiments. Differential expression analysis of miRNAs and mRNA between the pooled samples was performed using Affymetrix MiRNA Microarray technology version 4.0 (Affymetrix; Thermo Fisher Scientific, Inc.) (17) and GeneChip Human Transcriptome Array 2.0 (Thermo Fisher Scientific, Inc.) (18,19).

miRNA RT-qPCR. A TaqMan miRNA assay (Thermo Fisher Scientific, Inc.) was used to detect mature miRNAs using a Bio-Rad IQ5 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol, as previously described (6). The reactions were performed using the following parameters: 95°C for 2 min followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. RNU6-1 small nuclear RNA was used as an endogenous control for data normalization. Relative expression was calculated using the comparative threshold cycle method (20). Experiments were performed in triplicate. RT-qPCR analyses for the mRNA expression of target genes were performed using PrimeScript RT-PCR kits (Takara Biotechnology Co., Ltd., Dalian, China). The reactions were performed using the following parameters: 95°C for 2 min followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 20 sec, and then 72°C for 10 min. β-actin was used as an internal control (20). The primer sequences used are presented in Table I.

Table I. Primer sequences used in reverse transcription quantitative-polymerase chain reaction.

Gene	Direction	Primer sequence (5'- 3')
MTOR	F	ACAGCCCAGGCCGCATTGTC
	R	TCCAGGGACTCCGTCAGGCG
RICTOR	F	TCCGAAGAAATGTCAGTCAG
	R	GGCAGTGGCACAAGTGTT
STX10	F	ATCCCACATGACGAGTGGTG
	R	GGTCTGGGAACGAGAAGGTC
GRB2	F	CCAGAGCCAAGGCAGAAGAA
	R	AGTACTTCCCGGCTCCATCT
ATG9A	F	CGTGTGGGAAGGACAG
	R	GGCGCTTTCTCCACTC
ZEB1	F	TTAGTTGCTCCCTGTGCAGTT
	R	TAGGAGCCAGAATGGGAAAAG
CASP2	F	TCCAGCACAAAGGAGCTGATG
	R	GCTAGCACCCTCGGTTCTT
PRKCE	F	CACTGCAGTTGGACTTTGCC
	R	GACAGTGGACATGGGACTGG
ACTB	F	TTCCTTCTGGGCATGGAGTCC
	R	TGGCGTACAGGTCTTTGCGG

F, forward; R, reverse; MTOR, mechanistic target of rapamycin; RICTOR, RPTOR independent companion of MTOR complex 2; STX10, syntaxin 10; GRB2, growth factor receptor bound protein 2; ATG9A, autophagy-related protein 9A; ZEB1, zinc finger E-box binding homeobox 1; CASP2, caspase-2; PRKCE, protein kinase c ε; ACTB, β actin.

Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. In order to further understand the functions of miRNA and their targets, GO enrichment and KEGG pathway analysis for all annotated miRNAs and their targets were performed. Blast2GO was employed to store information from the GO (<http://www.geneontology.org/>) and KEGG (<http://www.kegg.jp>) pathway databases. All the sequences were identified by BLASTx searches against the GO protein database. A combined query was used in order to complete the GO annotation and pathway analysis against the GO and KEGG databases (21).

Transfection experiment. Mimic and control miR-96-5p were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfections were performed with DharmaFECT 1 transfection reagent (Thermo Fisher Scientific, Inc.). WPMY-1 cells were transfected with 100 nM mimic or miRNA control for 48 h, and then mRNA expression was subsequently assessed by RT-qPCR.

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using the Student's t-test for comparison of two groups in microarray analysis, and analysis of variance for comparisons of expression of selected aberrant miRNAs in RT-qPCR assay, using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

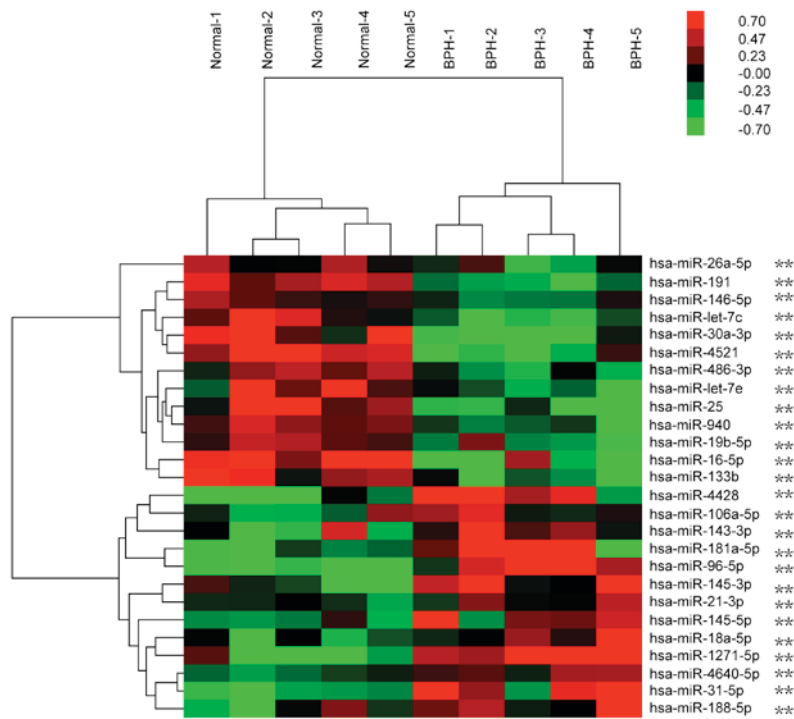


Figure 1. Differentially expressed miRNAs in the BPH and control groups. miRNA profiles of prostates differentiate between 5 patients with BPH and 5 healthy men. Both downregulated (red) and upregulated (green) miRNAs were identified (** $P < 0.001$). miRNA, microRNA; BPH, benign prostatic hyperplasia; hsa-miR, *Homo sapiens* microRNA.

Results

miRNA expression profiles in the prostate of patients with BPH and healthy controls. To investigate the miRNA expression profiles in the BPH and control group, array-based miRNA profiling of the prostate tissues from patients with BPH was performed. Of the 1,872 human miRNAs assayed, 23 miRNAs including miR-4428, miR-106a-5p, miR-96-5p and miR-1271-5p had higher expression levels in the prostate of patients with BPH when compared with healthy controls. The expression levels of 15 miRNAs including miR-16-5p, miR-19b-5p, miR-940, and miR-25 were reduced in the prostate of patients with BPH compared with healthy controls (Fig. 1). Of these 26 miRNAs, miR-96-5p and miR-1271-5p were significantly overexpressed, whereas miR-30a was significantly downregulated in the prostates of men with BPH.

Validation of miRNA dysregulation by miRNA RT-qPCR analysis. To confirm the microarray findings, the relative expression of each dysregulated miRNA was examined with miRNA RT-qPCR in all 30 BPH samples and the 5 healthy controls. The expression levels of hsa-miR-96-5p, hsa-miR-1271-5p, hsa-miR-21-3p, hsa-miR-96-5p, hsa-miR-181a-5p, hsa-miR-143-3p, hsa-miR-4428 and hsa-miR-106a-5p were significantly upregulated in the BPH group compared with healthy controls. The expression levels of hsa-miR-16-5p, hsa-miR-19b-5p, hsa-miR-940, hsa-miR-25, hsa-miR-486-3p, hsa-miR-30a-3p, hsa-miR-let-7c and hsa-miR-191 were significantly downregulated compared with the control (Fig. 2). These findings were in concordance with the microarray assay results. Notably, hsa-miR-96-5p

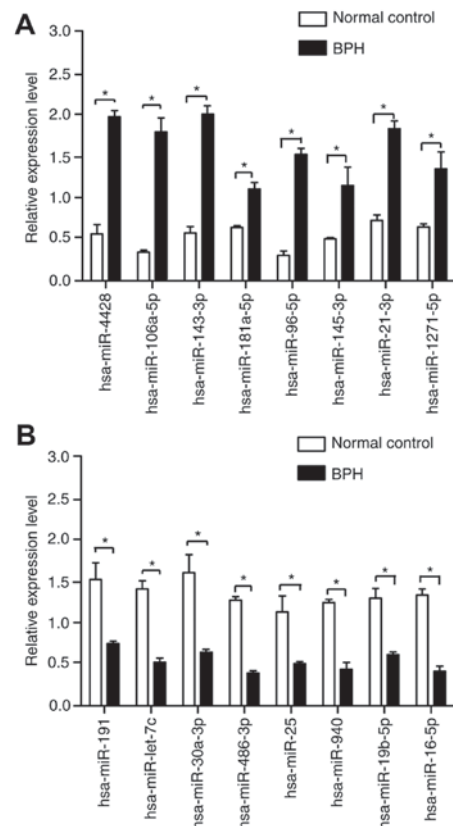


Figure 2. Validation of microarray data with miRNA reverse transcription quantitative-polymerase chain reaction. Expression levels of miRNAs were detected in 30 BPH cases and 5 healthy cases. (A) miRNAs with upregulated or (B) downregulated expression compared with the control group. * $P < 0.05$; miRNA, microRNA; BPH, benign prostatic hyperplasia; hsa-miR, *Homo sapiens* microRNA.

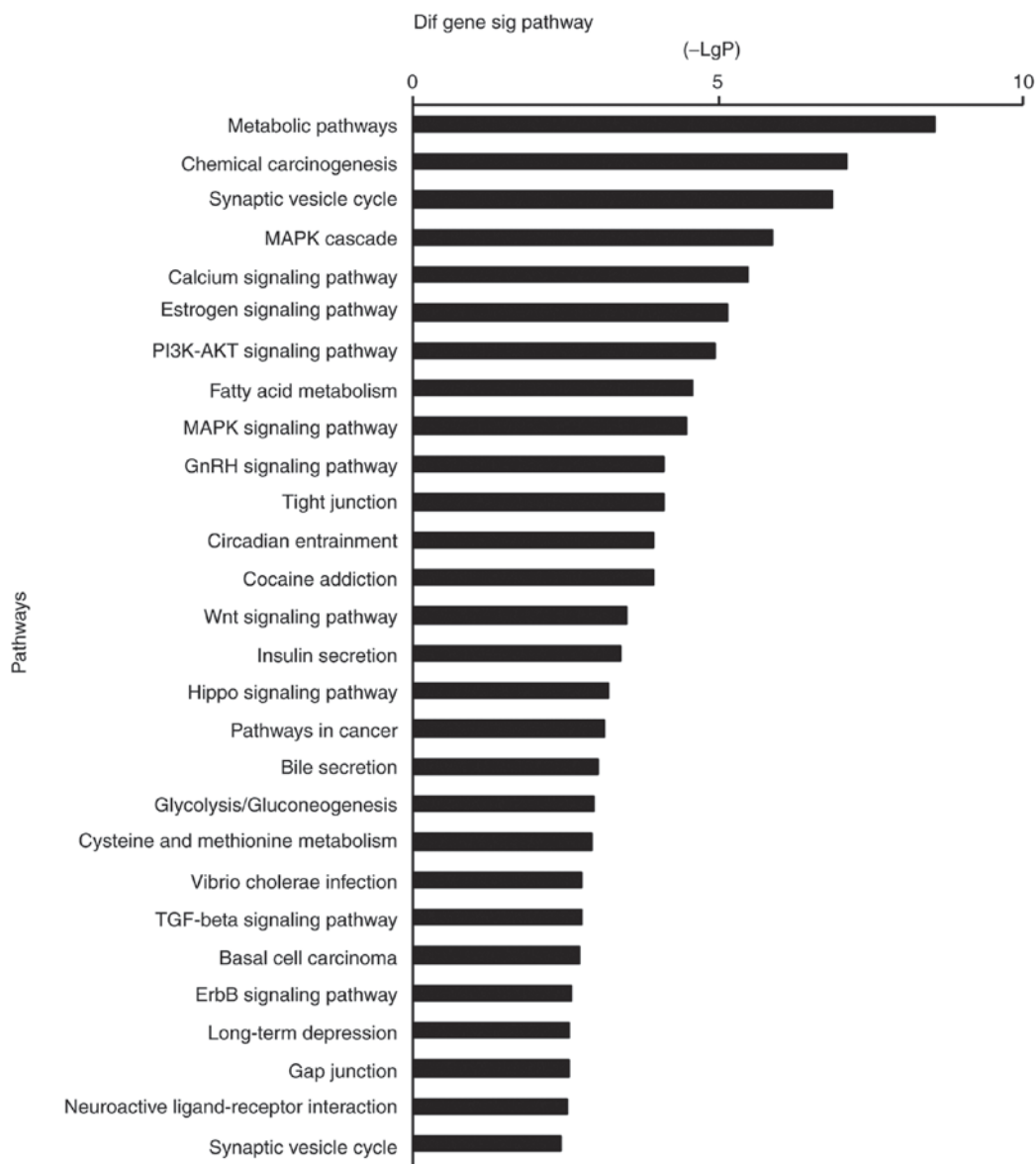


Figure 3. Pathway analysis based on miRNA target genes. The significant pathways downregulated by miRNAs are presented. -LgP is the negative logarithm of the P-value, with a larger -LgP indicating a smaller P-value. The pathways were likely upregulated due to the dysregulation of miRNAs in BPH. miRNA, microRNA; BPH, benign prostatic hyperplasia; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; GnRH, gonadotropin-releasing hormone; TGF- β , transforming growth factor β ; ErbB, epidermal growth factor receptor.

and hsa-miR-1271-5p have the same seed sequence and were classified in the same cluster.

Bioinformatics analysis. Gene ontology and KEGG pathway analysis of the signaling pathways regulated by miRNAs (as identified by RT-qPCR) revealed 47 upregulated signaling pathways and 28 downregulated signaling pathways in the BPH group compared to healthy controls. These regulated signaling pathways were involved in numerous cellular processes, including metabolic (pyrimidine, glutathione, phenylalanine, gluconeogenesis, and pyruvate), oncogenic (prostate cancer, colorectal cancer, and bladder cancer) and adhesive and signal transduction [estrogen, phosphoinositide 3-kinase (PI3K-AKT), Wnt, transforming growth factor β (TGF- β) and mitogen-activated protein kinase (MAPK)] signaling pathways. In addition, according to the enrichments of the significantly

downregulated signal genes, there were 28 downregulated signaling pathways (Fig. 3).

The miRNA-gene network of overlapping target genes identified the involvement of hsa-miR-96-5p (Fig. 4A). The major target genes of hsa-miR-96-5p were identified by TargetScan version 7.1 (www.targetscan.org), include mechanistic target of rapamycin (MTOR), RPTOR independent companion of MTOR complex 2 (RICTOR), syntaxin 10 (STX10), growth receptor bound protein 2 (GRB2), autophagy related 9A, zinc finger E-box binding homeobox 1 (ZEB1), caspase 2 (CASP2) and protein kinase c ϵ (PRKCE). In order to determine whether these genes were miR-96 target genes, WPMY-1 cells were transfected with miR-96-5p mimics or miR-96-5p control. As presented in Fig. 4B, miR-96 had an inhibitory effect on the mRNA expression level of these target genes, excluding GRB2. This verified that miR-96 may downregulate these target genes.

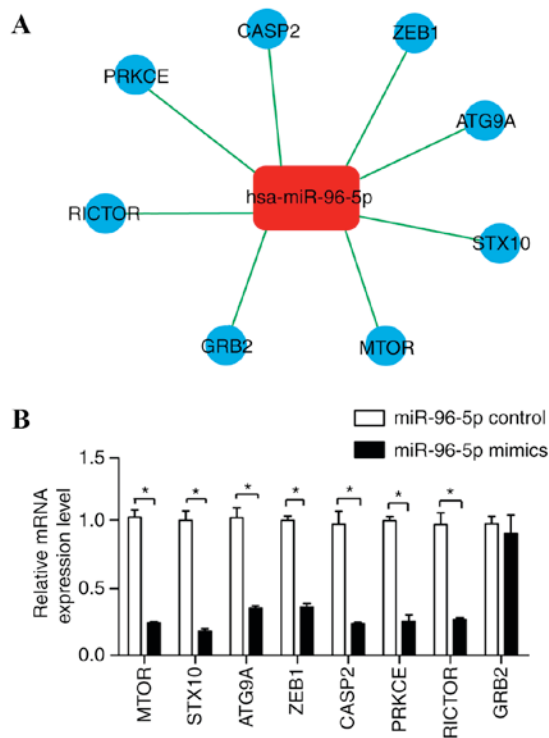


Figure 4. Major target genes of hsa-miR-96-5p. (A) miRNA-gene network. Red and blue box nodes represent upregulated miR-96-5p and downregulated genes, respectively. Edges represent the inhibitory effect of the miRNA on gene expression. (B) WPMY-1 cells were transfected with 100 nM miR-96-5p mimic or control at 48 h. The mRNA levels of these target genes were determined by reverse transcription quantitative-polymerase chain reaction. *P<0.05. miRNA, microRNA; hsa-miR, *Homo sapiens* microRNA; MTOR, mechanistic target of rapamycin; STX10, syntaxin 10; ATG9A, autophagy-related protein 9A; ZEB1, zinc finger E-box binding homeobox 1; CASP2, caspase-2; PRKCE, protein kinase c ϵ ; RICTOR, RPTOR independent companion of MTOR complex 2; GRB2, growth factor receptor-bound protein 2.

Discussion

miRNAs may regulate gene expression in various biological processes, including cancer, development, differentiation, signal transduction and cell maintenance (22,23). However, there are limited studies regarding differential miRNA expression in BPH. In the present study, miRNA expression profiles in the BPH and control group were evaluated to identify advanced BPH signatures. RT-qPCR was also used to identify some differentially expressed miRNAs. These findings are of note for future studies on BPH as miRNAs are a major determinant of cell proliferation.

The results of microarray analysis in the prostate specimens of 5 BPH and 5 healthy men revealed that 26 miRNAs were dysregulated. Among these differentially expressed miRNAs, miR-106a, miR-143, miR-96 and miR-1271 were overexpressed in the BPH group compared with controls, whereas the remaining 13 miRNAs were downregulated. A total of 8 upregulated miRNAs and 8 downregulated miRNAs were identified by RT-qPCR, suggesting that aberrantly expressed miRNAs may be involved in BPH development.

Altered miR-143, miR-106, miR-181, miR-21, miR-30a and miR-16 expression has been previously demonstrated to be involved in numerous types of cancer, including pancreatic,

colorectal, prostate and breast cancer (24-29). The microarray profile data of the current study revealed that these 6 miRNAs were also dysregulated in the BPH group, which may indicate their contribution to the pathogenesis of BPH and prostate cancer. Additionally, Viana *et al* (14) reported that miR-143 and miR-145 may be involved in the pathogenesis of BPH by inhibiting target genes, that include MAP kinase kinase kinase 4, KRAS proto-oncogene, GTPase and MAP kinase kinase kinase 3. Consistent with these findings, miR-143 was overexpressed in the BPH group compared with controls in the present study. Therefore, overexpression of miR-143 may contribute to BPH and further study of miR-143 dysregulation may provide an insight into the disease pathogenesis.

The miR-96-1271 cluster has been previously demonstrated to have different roles in various disease processes and may act as an oncogene or a tumor suppressor (30,31). Fendler *et al* (32) identified that dysregulation of the miR-96 cluster in prostate cancer resulted in forkhead box O1 inhibition, demonstrating its function as an oncogene. In the present study, several target genes of the miR-96-1271 cluster were predicted *in silico*, including MTOR, STX10, RICTOR, ZEB1, CASP2 and PRKCE. To date, many genes have been experimentally validated as targets of the miR-96-1271 cluster, including receptors, transcriptional regulatory proteins, kinases and oncogenes (33-36). In the present study, WPMY-1 cells were transfected with miR-96-5p mimic or control and the mRNA expression of these genes was detected with RT-qPCR. MiR-96 was demonstrated to have an inhibitory effect on the mRNA expression level of these target genes, excluding GRB2.

Gene ontology enrichment analysis identified target genes that were differentially regulated by miRNAs and also had different functions in biological processes between the BPH and control group, including cell proliferation, differentiation, adhesion, cycle, apoptosis and metabolism. In the current study, 47 upregulated and 28 downregulated pathways were identified to have potential involvement in BPH development. These signal pathways were abundant among the significantly enriched pathways, including metabolic (pyruvate, phenylalanine, and gluconeogenesis), oncogenic (prostate, colorectal and bladder cancer), and adhesive and signaling (estrogen, PI3K-AKT, Wnt, TGF- β and MAPK) pathways. These results demonstrate that miRNAs may influence several signaling pathways that affect BPH progression.

In summary, dysregulated miRNA expression may be involved in the progression of BPH through miRNA target gene inhibition. The specific mechanism of miRNA involvement in BPH remains to be determined. However, the present study provides a foundation for further study of the role of miRNAs in BPH.

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