miRNA Regulation Network Analysis in Qianliening Capsule Treatment of Benign Prostatic Hyperplasia

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Objective. The objective of this study was to evaluate the molecular mechanism by which Qianliening capsule (QC) treats benign prostatic hyperplasia (BPH).

Methods. Benign prostatic hyperplasia epithelial cell line BPH-1 was treated with 0, 1.25, 2.5, and 5 mg/mL QC for 48 h, respectively. Evaluation of cell viability and observation of morphologic changes of BPH-1 cell gene expression and miRNA expression profiles were analyzed. Real-time quantitative PCR was used to confirm changes in miRNA and gene expression. GO and KEGG pathway-based approaches were used to investigate biological functions and signaling pathways affected by differentially expressed mRNAs.

Results. QC inhibited BPH-1 cell proliferation. Differential expression of 19 upregulated and 2 downregulated miRNAs was observed in QC-treated BPH-1 cells compared to untreated control cells. 107 upregulated and 71 downregulated genes were identified between the two groups. Significantly enriched signaling pathways based on deregulated mRNAs were mainly involved in regulation of cell proliferation, apoptosis, and so on. Additionally, miRNA-miRNA network analysis integrated these miRNAs and genes by outlining interactions of miRNA and related genes.

Conclusion. The study was the first report of differentially expressed miRNA and mRNA in QC-treated BPH-1 cells.

1. Introduction

Benign prostatic hyperplasia (BPH) is a pathological overgrowth of the human prostate, a condition that affects a majority of men older than 50 years [1]. Most patients with BPH experience increased resistance in urinary flow leading to urinary tract symptoms (LUTS) including urinary hesitancy, frequent urination, urgency, thin urine flow, and bladder stones [2]. These symptoms greatly affect the physical and mental health of patients as well as their quality of life. Unfortunately, the cellular and molecular mechanisms contributing to BPH development and progression remain unclear.

Microribonucleic acid (miRNA), a 19–24 noncoding family of nucleotide (nt), is processed from 70 to 100 nt double-stranded hairpin precursors by RNase III dicer and endogenously expressed in the RNA-induced silencing complex within the cytoplasm [3]. miRNA recognizes the 3′-untranslated region of target mRNA(s) with imperfect complementarity which causes translational repression or mRNA cleavage [4]. Currently, growing evidence highlights the importance of miRNAs in association with pathophysiologic processes including abnormal cell proliferation and differentiation, invasion, metastasis, and the poor prognosis of various cancers and relative diseases [5–9]. Therefore, it is plausible that certain miRNAs participate in the pathogenesis of BPH, which constitutes the rationale for our research.

The mainstay of pharmacotherapy for BPH is a combination treatment of 5α-reductase inhibitors that regulate levels of 5-dihydrotestosterone (DHT) and α-adrenergic blockers that inhibit α-adrenergic receptors. However, these
medications may induce undesired side effects such as ortho-
static hypotension [10–14]. Qianliening capsule (QC) is a tra-
ditional Chinese medicine formulation consisting of wine
rhubarb, leech, Milkvetch root, Achyranthes aspera, and dod-
ders. These components together confer QC properties of
heat-clearing, detoxification, promotion of blood circulation,
removal of blood stasis, tonifying the kidney, and nourishing
vitality (replenishing the kidney qi in Chinese) [15, 16].
Previous studies have demonstrated that QC has significant
therapeutic effects on BPH [16–18]. In clinical trials, QC
clearly improved a series of lower urinary tract symptoms
(LUTS) in BPH patients, such as frequency of urination,
urinary urgency, thin urine flow, incontinence, and other
voiding disorders [19]. Our preliminary study in a rat model
of BPH showed that QC significantly decreased prostatic vol-
ume and weight, inhibited prostatic hyperplasia, attenuated
abnormal serum levels of estrogen and androgen, regulated
the expression of estrogen receptor (ER), androgen receptor
(AR), and related mRNA, inhibited the EGFR/STAT3 pathway,
and reduced expression of proproliferative PCNA, cyclin
D1, and CDK4 proteins [15–19]. Moreover, QC effectively
inhibited proliferation and promoted apoptosis in human
benign prostatic hyperplasia epithelial cells and prostate cells
[20, 21]. To more fully clarify the mechanistic effects of
QC therapy in the treatment of BPH, we performed the
present study to examine the effects of QC on expression of
specifically expressed miRNAs, genes, and relevant signaling
pathways in our rat model of BPH.

2. Materials and Methods

2.1. Materials and Reagents. Fetal bovine serum (FBS), Ros-
well Park Memorial Institute 1640 (RPMI 1640) medium,
penicillin-streptomycin, and trypsin-EDTA were purchased
from Life Technologies (Carlsbad, CA, USA). A cell prolif-
eration WST-1 assay kit was obtained from Roche Applied
Science Gmbh (Mannheim, Germany). TRIzol reagent was
purchased from Promega Corporation (Madison, WI, USA). All images were acquired at 400x
magnification.

2.2. Preparation of QC. Qianliening capsule (QC, Fujian,
China, FDA approval number: Z09104065) was provided by
the Academy of Pharmacology of Fujian Chinese Medical
University. The drug powder inside the capsule was dissolved
in distilled water and stored at −20°C. Working concen-
trations of QC were prepared by diluting the stock solution in
culture medium.

2.3. Cell Culture. Benign prostatic hyperplasia epithelial
(BPH-1) cells were obtained from Xiangya Cell Center, Uni-
versity of Zhongnan (Hunan, China), and grown in RPMI
1640 medium. RPMI 1640 was supplemented with 10% (v/v)
FBS and 100 units/mL penicillin and 100 µg/mL strepto-
mycin. All cells were cultured at 37°C and 5% CO₂ under
humidity.

2.4. Evaluation of Cell Viability by WST-1 Assay. The influ-
ence of increasing QC concentration on the viability of BPH-1
cells was determined using a cell proliferation reagent WST-
1 kit. Briefly, BPH-1 cells were harvested from exponential
phase cultures growing in RPMI 1640 with 10% FBS, counted,
plated in 96-well flat-bottomed microtiter plates (100 µL cell
susensions, 2.5 × 10⁴ cells/mL), and treated with medium
containing various concentrations (0 mg/mL, 1.25 mg/mL,
2.5 mg/mL, and 5 mg/mL) of QC. After 48 h, 10 µL WST-1
was added to each well and the reaction mixture was incubated
at 37°C in a 5% CO₂ atmosphere for 0.5 h. Sample absorption
was measured under a wavelength of 450 nm using a spec-
trophotometer (Bio Tek Model ELX800, USA), and the results
were compared as percentages of control cells.

2.5. Observation of Morphologic Changes. BPH-1 cells were
seeded into 6-well plates at a density of 1.0 × 10⁵ cells/well in
2 mL medium. The cells were treated with various concen-
trations (0, 1.25, 2.5, 5 mg/mL) QC for 48 h. Cell morphology
was observed under a phase-contrast microscope (Leica,
Mannheim, Germany). All images were acquired at 400x
magnification.

2.6. RNA Extraction for Microarray and Real-Time PCR Anal-
ysis. BPH-1 cells were seeded into 75 cm² flasks at a density
of 5 × 10⁴ cells/flask in 15 mL medium. The cells were treated
with 2.5 mg/mL QC for 48 h and total RNA, including small
RNA, was isolated with TRIzol reagent. RNA purity and con-
centration were determined via OD 260/280 readings using
a spectrophotometer (Nanodrop 2000c). Concerning differ-
ential miRNAs and mRNAs with comparative microarray-
determined expression levels over 2.0 or below 0.5, real-
time PCR assays were utilized to detect and quantify the
differential pre-miRNAs and mRNAs as previously described
[22, 23] using SYBR Green dye. U6 was used as an internal
control. The threshold cycle (CT) is defined as the fractional
cycle number at which fluorescence passes a fixed threshold.
The miRNA and mRNA expression levels were normalized to
U6, relative expression was calculated using the comparative
ΔΔCT method, and values were expressed as 2^−ΔΔCT [24].

2.7. miRNA Expression Microarray Analysis. A GeneChip
miRNA 3.0 array (Affymetrix, Santa Clara, CA) was hybrid-
ized using 500 ng total RNA per standard Affymetrix pro-
ocols. The same RNA preparations used in the miRNA
microarray analysis were used for miRNA array analysis. Data
extraction was completed using Affymetrix Command Con-
sole software. Raw data was analyzed by the following workflow: background detection, RMA global background
correlation, quantile normalization, median polish, and log 2
transformation with miRNA QC tool software.

2.8. Gene Expression Microarray Analysis. Microarray analy-
sis was accomplished by hybridization to a GeneChip Prim-
View human gene expression array per manufacturer’s
instructions (Affymetrix, Santa Clara, CA). Genes that
underwent at least a 2-fold change between treatment groups
were selected for real-time PCR validation.
2.9. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes Pathway Analysis Based on Differentially Expressed mRNAs. Gene ontology (GO) (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (http://www.genome.ad.jp/kegg/) were used to investigate biological functions and signaling pathways affected by differentially expressed mRNAs [25–27]. A $P$ value less than 0.05 was considered statistically significant. Meanwhile, we constructed the miRNA-mRNA regulatory network basis on different miRNAs and their targets; the miRNA-mRNA interaction network, representing critical miRNAs and their targets, was established according to miRNA degree [28]. Networks of miRNA and genes from differentially expressed miRNA and mRNA in the two groups were visualized with the Cytoscape tool [28].

2.10. Statistical Analysis. Data are presented as means ± SD for the indicated number of independently performed experiments and analyzed using the SPSS software package for Windows (version 16.0). Statistical analysis of the data was performed with Student’s t-test and ANOVA. Differences with $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. QC Inhibits Proliferation of BPH-1 Cells. The effect of QC on BPH-1 cell viability was determined by the WST-1 assay. As shown in Figure 1, treatment with 1.25–5 mg/mL QC for 48 h reduced cell viability by 50%–80% in a dose-dependent manner compared with untreated control cells ($P < 0.05$). To further verify these results, we evaluated the effect of QC on BPH-1 cells confluence via phase-contrast microscopy. QC treatment gradually decreased monolayer confluence with increasing drug concentration (Figure 2). Taken together, these data demonstrate that QC inhibited BPH-1 cell proliferation.

3.2. miRNA Expression Profiling. The GeneChip miRNA array version 3.0 (Affymetrix) was used to profile miRNAs that were differentially expressed between BPH-1 cells treated with QC (2.5 mg/mL) and control cells. All of the 1733 miRNAs that were probed, 21 were differentially expressed in QC-treated BPH-1 cells (Table 1). These are shown visually in a cluster analysis (Figure 3(a)). 19 of 21 were overexpressed whereas 2 of 21 were underexpressed. The miR-34 family (including miR-34a and miR-34c), miR-3185, miR-663, and miR-125b, are involved in apoptosis and proliferation [29–34]; miR-122 and miR-1231 are associated with liver disease [35–37], and miR-3663 is associated with skin or smooth muscle cancer [38, 39]. However, the biological functions of the other miRNAs that significantly changed in the miRNA array have not been reported previously and require further study.

3.3. Gene Expression Profiling. To gain insight into the mechanisms of QC action, we conducted gene expression profiling by GeneChip PrimeView human gene expression array, as shown visually via cluster analysis (Figure 3(b)). Significant changes in 178 genes were observed between QC-treated BPH-1 cells and controls, of which 107 genes were upregulated and 71 genes were downregulated (Table 2). Genes that were identified as differentially expressed exhibited $>2.0$-fold change between the BPH-1 cells treated with QC and control cells.
The top 10 different genes are shown.

3.4. Validation of Differentially Expressed miRNAs and Genes. Real-time PCR was used to validate the microarray analysis findings. The relative expressions, expressed as percent change from controls of the five chosen miRNAs (miR-34a, miR-122, miR-34c, miR-4703, and miR-1972), are shown in Figure 4(a). These data confirmed that miR-34a, miR-122, miR-34c, and miR-4703 were increased in QC-treated BPH-1 cells whereas miR-1972 expression was decreased (\( P < 0.05 \) for all). Meanwhile, we randomly selected 8 different genes (CYP1B1, ALDN3, CYP1A1, ANKRD1, KRTAP2-3, and SPINK2), and these data confirmed that CYP1B1, ALDN3, CYP1A1, and ANKRD1 were overexpressed in two groups whereas KRTAP2-3 expression was decreased (\( P < 0.05 \) for all) (Figure 4(b)). SPINK2 expression, which was not significantly different from controls, was also confirmed not to be significantly different between the two groups.

3.5. GO and KEGG Pathway Analysis of Deregulated mRNAs. GO analysis showed that the differentially expressed mRNAs between QC-treated BPH-1 cells and the control group were significantly enriched in oxidation reduction, regulation of cell proliferation, immune response, regulation of apoptosis, regulation of cell death, and so forth. KEGG pathway analysis indicated that the deregulated mRNAs between the two cell groups were mainly involved in metabolism of xenobiotics by cytochrome P450, steroid hormone biosynthesis, drug metabolism, and TGF-beta signaling pathway (\( P \) value < 0.05 after multiple testing corrections) (Tables 3-4). Additionally, miRNA-mRNA network analysis integrated these miRNAs and genes by outlining the interactions between them (Figure 5).
Figure 3: Cluster analysis of differentially expressed miRNA and mRNA with and without QC treatment. (a) miRNA signature in BPH-1 cells treated with QC and controls. (b) mRNA signature in BPH-1 cells treated with QC and controls. Signal intensity was expressed as a log2 ratio between QC treatment and controls. Bright blue, underexpression; white, no change; bright red, overexpression. P < 0.05.

4. Discussion

Qianliening capsule (QC) is a traditional Chinese formulation that has long been used to clinically treat benign prostate hyperplasia (BPH). However, BPH is a complex disease as its pathogenesis and progression are associated with multiple factors, genes, and signal transduction pathways, all of which are further highly regulated by an miRNA regulatory network. As such, much effort has been placed on understanding the mechanisms underlying the disease process and
treatment mechanisms of QC in BPH. Research on miRNAs in various diseases is still in its infancy, although exciting findings including the link between miRNAs and prostate cancer have recently been reported [40]. To our knowledge, however, there have been no reports on the association between miRNAs and any spectrum of BPH. In order to identify potentially unique miRNA expression profiles, we jointly employed miRNA and gene microarray analysis to detect aberrantly expressed miRNA and mRNA in BPH-1 cells. Hundreds of miRNAs have been shown to play important roles in regulating gene expression through degradation of mRNA or repression of translation in a variety of model systems [41, 42]. Microarray gene expression profiling has improved our understanding of BPH biology and allowed the development of multigene “signatures” to predict outcome and response to systemic therapies [32]. Through comprehensive array profiling and analysis of miRNA and gene expression levels, we have identified putative candidate genes and pathways that mediate QCs effects in BPH. Genes differentially expressed in response to QC treatment included those involved in cell growth, proliferation, and apoptosis, common characteristics in hyperplastic diseases.

Our preliminary research showed that QC could inhibit proliferation and promoted apoptosis in vivo and in vitro [20, 21]. According to our miRNA array results, most of the miRNAs have previously been shown to be associated with apoptosis, proliferation, and metabolism. For example, the miR-34 family, including miR-34a and c, has been known to regulate several cellular events, including the cell cycle, cell migration, and apoptosis [29, 30]. miR-663 is involved in the TGF-β signaling pathway [32, 33]. miR-3185 likely plays an important role in regulating MAPK signaling [31]. miR-122 is suggested to regulate many target genes in lipid and cholesterol metabolism [35, 36]. Interestingly, among 242 altered genes identified by our gene microarray experiment, more than 30 genes were involved in apoptosis and proliferation. Their cellular functions encompass regulation of cell proliferation, cell cycle, and cell death. More than 10 genes were involved in metabolism (metabolism of xenobiotics and drug metabolism). The miRNA-mRNA interaction network analysis further integrated bioinformatic findings and then outlined the primary different miRNAs and their major target gene(s). It is of great interest to determine in future studies whether these same sets of miRNAs and their target genes

| Pathway ID | Definition | Gene count | P value |
|------------|------------|------------|---------|
| hsa00980   | Metabolism of xenobiotics by cytochrome P450 | 10         | 1.87E – 09 |
| hsa00140   | Steroid hormone biosynthesis | 6          | 5.04E – 05 |
| hsa00898   | Drug metabolism | 5          | 0.00223 |
| hsa04350   | TGF-beta signaling pathway | 4          | 0.04332 |

Enriched KEGG pathways were used for analysis of the differentially expressed mRNAs between two groups; P values after multiple testing corrections <0.05.
Table 4: List of enriched GOs of differentially expressed mRNAs in BPH-1 cells treated with QC (2.5 mg/mL) and control cells.

| ID         | Definition                           | Gene count | P value       |
|------------|--------------------------------------|------------|---------------|
| GO:0055114 | Oxidation reduction                  | 18         | 1.20E–06      |
| GO:0042127 | Regulation of cell proliferation     | 15         | 8.27E–04      |
| GO:0006955 | Immune response                      | 14         | 7.61E–04      |
| GO:0042981 | Regulation of apoptosis              | 14         | 0.002994      |
| GO:0043067 | Regulation of programmed cell death  | 14         | 0.003365      |
| GO:0010941 | Regulation of cell death             | 14         | 0.003694      |
| GO:0012625 | Death                                | 12         | 0.009416      |
| GO:0010033 | Programmed cell death                | 11         | 0.00846       |
| GO:0043065 | Positive regulation of apoptosis     | 10         | 0.002571      |
| GO:0043068 | Positive regulation of programmed cell death | 10 | 0.002694 |
| GO:0009611 | Response to wounding                 | 10         | 0.009872      |
| GO:0006915 | Apoptosis                            | 10         | 0.021064      |
| GO:0030182 | Neuron differentiation               | 9          | 0.009842      |
| GO:0042493 | Response to drug                     | 8          | 6.67E–04      |
| GO:00444092| Negative regulation of molecular function | 8     | 0.007664 |
| GO:0022493 | Cell cycle phase                     | 8          | 0.022599      |
| GO:0008284 | Positive regulation of cell proliferation | 8     | 0.022599 |

Enriched GOs were used for analysis of the differentially expressed mRNAs between two groups; P values after multiple testing corrections <0.05.

Figure 5: Interaction network of upregulated genes and downregulated miRNAs/mRNAs in BPH-1 cells treated with QC (2.5 mg/mL) and control cells. Blue box nodes represent miRNA, and red cycle nodes represent mRNA. Edges show the inhibitory effect of miRNA on mRNA. The miRNA-mRNA network was visualized by Cytoscape tool.
could be biomarkers that are associated with mechanisms by which QC treats BPH.

**Abbreviations**

BPH: Benign prostatic hyperplasia  
QC: Qianliening capsule  
miRNAs: MicroRNAs.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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