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The expression of androgen-responsive genes is up-regulated in the epithelia of benign prostatic hyperplasia

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Abstract

BACKGROUND—Benign prostatic hyperplasia (BPH) is one of the most common diseases among aging men in the United States. In addition to aging, the presence of androgens is another major risk factor in BPH development. However, whether androgen signaling is altered in BPH remains unclear. To determine androgen signaling in BPH, we characterized the expression of 4 different androgen-responsive genes, Eaf2/U19, ELL2, FKBP5, and PSA, in BPH and adjacent normal glandular epithelial cells.

METHODS—A set of 17 BPH specimens were from patients over 60 years of age with clinical symptoms of BPH. Laser-capture microdissection (LCM) was used to isolate glandular epithelial cells from BPH areas and adjacent normal areas, separately. LCM isolated cells from individual specimens were lysed and RNA isolation, reverse transcription, and real-time PCR were performed using CellsDirect™ One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA).

RESULTS—All of the assayed genes displayed increased expression from ~2-fold to ~6-fold, in BPH as compared to the adjacent normal epithelial cells. We also generated a composite androgen response index based on the expression levels of the 4 genes, which provides a reliable readout for overall androgen action. Our study showed that the composite androgen response index in BPH is ~4-fold as compared to that in the adjacent normal tissues.

CONCLUSIONS—Androgen signaling is significantly elevated in BPH relative to the adjacent normal prostate. Understanding the mechanisms causing elevated androgen signaling may lead to novel approaches for prevention and/or treatment of BPH.

Keywords

human; BPH; androgens; androgen-responsive genes

Introduction

Benign prostatic hyperplasia (BPH) contributes substantially to the disease burden in aging men. The prevalence of BPH increases linearly with age, from almost nil in the third decade of life to almost 100% in the ninth decade (1–3). While BPH is not synonymous with clinical disease, prostatic enlargement with underlying hyperplasia is a major determinant of lower urinary tract symptoms (LUTS) (2,4,5).

The socio-economic costs of BPH are tremendous. Three out of four men over the age of seventy will have some degree of LUTS, and more than half of these will have moderate to severe symptoms (3). The annual medical expenditure of BPH in the United States, including medical services and pharmaceuticals, is about \$4 billion, ~twice that of prostate cancer (CaP) (6–8). Although BPH is almost never fatal, the morbidity of LUTS, and the potential complications due to BPH place a great burden on patients as well as the health care system (2,4,5,7).

BPH differs from prostate cancer in localization, clinical presentation, and management considerations, and is believed to have a different etiopathogenesis. Yet both are strongly associated with aging and androgens (2,9). BPH is a hyperplastic process (10), but despite having an increased number of glandular elements, these cells do not progress to CaP. Clearly, the multiplicative effect of aging and androgens need to be analyzed in the context of these very different disease responses.

One strategy to explore this would be to study the expression of various genes, particularly androgen-responsive genes. Although there are many studies examining gene expression patterns in prostate cancer, very few look at BPH epithelia. The few studies that have focused on the transcriptome changes in both BPH as well as CaP have revealed varied and interesting patterns of gene expression. Differential expression of genes involved in cell regulation, signaling/transcription, and stromal composition have been reported (11–14). Differences in results may be due in part to tissue heterogeneity caused by mixing of normal and diseased cells, epithelial and stromal components, mixing of samples from different prostate zones, and sample pooling (11,12,14). The slow-growing nature of BPH implies the likelihood that modest transcriptome changes may be important in the primary pathology of this disease (14). These changes may be difficult to detect in the analysis of heterogeneous samples mentioned above.

As mentioned previously, androgens and aging play essential roles in the development of BPH and CaP. Men who are castrated before puberty do not develop BPH or CaP (15). Androgens are required for growth, development, and differentiation of the prostate, and the continued presence of androgens through aging is necessary for the development of both BPH and CaP (15,16). Therefore, the androgen signal signature, especially when viewed from a long term perspective, may provide important clues regarding the pathogenesis of BPH. Data on androgen signaling in BPH are limited and the role of androgen signaling in BPH pathogenesis remains unclear. The expression of the androgen receptor has been reported to be both increased and decreased in BPH cells (14,16). These differences could be due, in part, to the selection and measurement biases outlined above. Additionally, there is a paucity of data in clinical specimens which explore androgen response gene expression in BPH and contrast it to that in normal adjacent tissue.

This study was designed to examine potential differential androgen-responsive gene expression in BPH as compared to adjacent normal tissue and to establish reproducible gene expression data. The genes investigated in our study were the androgen receptor (AR) and 4 androgen-responsive genes, consisting of prostate specific antigen (PSA), FKBP5, Eleven-nineteen lysine-rich leukemia gene 2 (ELL2), and up-regulated gene 19/ELL-associated factor 2 (U19/EAF2). PSA is a secretory gene widely used as a tool for diagnosis, prognostication, and follow up for prostate cancer (17,18). FKBP5 is an immunophilin with reported functions in cellular trafficking of steroid-hormone receptors (19,20). ELL2 is a member of ELL family proteins and could function as an RNA polymerase II elongation factor, is also regulated by androgens in the prostate (21,22). U19/EAF2 is a positive regulator of the RNA polymerase II elongation activity of ELL proteins and has been shown to have tumor suppressive activities in vitro and in vivo (23–26). Characterizing the expression of these genes will provide insights into possible alterations in androgen action in these prostatic diseases. We restricted our studies to tissue

samples which correlated with clinical significance by including BPH samples only from patients over 60 years of age with clinical symptoms of LUTS and who also underwent prostatectomy because of BPH. Using laser-capture microdissection and quantitative RT-PCR, we compared the expression levels of AR and the above mentioned 4 androgen-responsive genes in BPH epithelial cells with that in the adjacent normal epithelial cells.

Materials and Methods

Tissue acquisition and laser-capture microdissection

Human BPH specimens were obtained from the Health Sciences Tissue Bank at the University of Pittsburgh Medical Center under approval by the UPMC Institutional Review Board following a standard protocol. All the specimens were from patients over 60 years of age with clinical symptoms of BPH and who also underwent prostatectomy because of BPH. No incidental foci of carcinoma were present in this cohort. Prostate specimens were embedded in Tissue Tek OCT (Sakura Finetek U.S.A, Torrance, CA) and stored at -80°C until sections were cut at $8\mu\text{m}$. Sections were mounted on PEN membrane slides (Leica Microsystems, Wetzlar, Germany). Serial specimens were histologically reviewed by a pathologist to identify specific pathological areas. Tissue sections were fixed in 70% EtOH for 30 seconds and rinsed with double distilled H₂O. Sections were stained with Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO) for 30 seconds, rinsed with double distilled H₂O and placed in 0.01% Eosin (Acros Organics, Geel, Belgium) for 5 seconds. Prostate specimens were dehydrated for 30 seconds twice in 95% ethanol, 30 seconds in 100% ethanol, and 2 minutes in Xylenes. The tissue was air-dried and LCM was performed using the Leica LMD6000 (Leica Microsystems, Wetzlar, Germany). Approximately 2000–5000 excised cells were captured into 0.5ml Eppendorf tube caps.

RNA isolation and real-time PCR analysis

Captured individual tissue specimens were lysed and RNA isolation, reverse transcription, and real-time PCR were performed using CellsDirect™ One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). Gene-specific primers and Taqman probes cross exon/exon junctions and were designed with Primer 3 Software (27) (Primer3, Totowa, NJ) (Table 1). Probes contained FAM fluorophore and TAMRA quencher. TBP was purchased from Applied Biosystems (Foster City, CA). The primer and probe combination were optimized within suitable ranges for efficiency and correlation coefficient using standard curve dilutions and data output on the ABI Step-One Plus thermocycler (Applied Biosystems, Foster City, CA). We also performed Taqman real-time PCR and showed that all of the primers used in our study only amplified cDNA and not genomic DNA. The reactions were analyzed in triplicate and normalized to GAPDH. All oligos were tested to ensure genomic DNA was not amplified. Real-time PCR was performed on a Bio-Rad IQ5 (Bio-Rad Laboratories, Hercules, CA) or ABI Step-One Plus (Applied Biosystems, Foster City, CA). Real-time PCR data were analyzed by ΔCp (crossing point) method as $R=2^{[\text{Cp sample} - \text{Cp control}]}$ (28) to generate the relative expression ratio (R) of each target gene relative to GAPDH. We typically used 2000–5000 isolated glandular epithelial cells per sample in the real-time qPCR assays (Fig 1). However, we were able to perform reverse transcription and qPCR from as few as 300 isolated cells. All of our primer-probe combinations showed efficiencies of greater than 90% (Table 1).

Statistics were performed using Graphpad Software (Graphpad Software, La Jolla, CA) for paired student's t test for means. Descriptive statistics were also generated by Graphpad Software.

Results

The expression of AR is elevated in BPH epithelial cells relative to the adjacent normal epithelial cells

According to literature, AR protein level in BPH specimens is about 47% higher than that the normal prostate tissues (16). To determine if AR expression in BPH epithelia is higher than that in the adjacent normal controls, we used LCM coupled with real-time RT-PCR. Fig. 2 shows that the expression of AR mRNA is about 1.9-fold relative to that in the adjacent normal control epithelial cells. The difference was not statistically significant, mainly due to large variations in different specimens. However, our finding is consistent with the result in the literature.

The expression of androgen-responsive genes is elevated in BPH epithelial cells relative to the adjacent normal epithelial cells

Androgen action is mediated through AR, a ligand-dependent transcription factor that regulates the expression of androgen-responsive genes. Thus, the best readout for AR activity is the mRNA levels of androgen-responsive genes, particularly those that are up-regulated by androgens in the prostate. The genes that are down-regulated by androgens are not suitable in this study because their expression is very low or not detectable in intact prostate.

Our study tested the expression of 4 androgen-responsive genes, PSA, U19/Eaf2, ELL2, and FKBP5, in clinical BPH specimens resected from 17 different patients. Fig. 3 shows that the mRNA levels of PSA, U19/EAF2, ELL2, and FKBP5 were upregulated 5.4-fold ($p=.01$), 2.2-fold ($p=.01$), 6.0-fold ($p=.05$), and 3.4-fold ($p=.05$), respectively, in BPH epithelial cells relative to that in the adjacent normal epithelial cells. This result indicates that the expression of these androgen-responsive genes is elevated in BPH epithelial cells relative to the normal adjacent control.

TATA box binding protein (TBP) was used as a control for global changes and does not show transcription level changes. Our findings suggest that the expression of other androgen-responsive genes in BPH epithelial cells should also be elevated relative to the adjacent normal control.

Composite androgen-responsive index provides a readout for overall androgen signaling

The use of an individual androgen-responsive gene as readout may not be an optimal indicator of overall androgen signaling. There were significant variations in the expression of individual androgen-responsive genes in different specimens. To provide a better description of overall androgen response in the prostate, we decided to develop a way to include all 4 androgen response gene expression data collected in this study into a composite androgen-responsive index of multiple androgen-responsive genes. The composite androgen response index should be a more accurate readout for overall androgen response than a single androgen-responsive gene and mitigate the large variation in some androgen response genes. In our studies, the composite androgen index was determined by taking the sum of the normalized levels of 4 different androgen-responsive genes and divided that by 4 (Fig. 4A). The following is the formula used in the calculation:

$$\text{Androgen Index} = [\text{Normalized Relative Expression of Gene 1 (N.R.E. 1)} + \text{N.R.E. 2} + \dots + \text{N.R.E. N}] / N$$

where N is the number of androgen-response genes used in the calculation. Since we used 4 different androgen-responsive genes in the present study, the N was 4 in Fig. 4A. Normalized

relative expression of a gene is a value of relative expression of the gene divided by the mean of relative expression of that gene in the normal areas adjacent to BPH.

The composite androgen-response index minimizes the variability associated with using individual androgen-response genes as the readout (Fig. 4B). The ratio of the composite index in BPH relative to the normal adjacent is expected to remain ~4-fold as seen in the composite androgen index, when additional androgen-responsive genes are included. As expected, the coefficient of variation decreased when more genes were used in the composite index calculation (Fig. 4C). When 4 genes were used, the coefficients of variation were 0.433 and 0.505 for BPH and adjacent normal cells, respectively. Thus, the composite androgen-response index provides an accurate and sensitive readout for overall androgen response in human prostate specimens.

Discussion

This study reports the finding that all of the assayed 4 androgen-responsive genes, PSA, FKBP5, U19/Eaf2, and ELL2, displayed elevated (2- to 6-fold) expression in BPH cells relative to the adjacent normal epithelial cells in clinical specimens. PSA and FKBP5 are two well characterized androgen-responsive genes abundantly expressed in the prostate (17,19,20). U19/Eaf2 is a newly discovered tumor suppressor that regulates prostate growth (23,25,26). ELL2 is a binding partner of U19/Eaf2 (21,23). These 4 genes consist of very different primary sequences and are expressed at different levels. One thing in common for these genes is that they are all up-regulated by androgens in the prostate. Since many other androgen-responsive genes are regulated by AR similarly, it is likely that most androgen-responsive genes would have similar up-regulation in BPH cells. Thus, characterization of these 4 different genes should provide insights into the overall androgen response in BPH pathogenesis.

Using a single androgen-responsive gene as readout is associated with high variations in its expression levels in different specimens and may not reflect the overall androgen response adequately. Thus, we utilized a composite index of normalized expression of the 4 different androgen-responsive genes, which we have termed the “composite androgen response index”, as the readout for androgen response. A major advantage of using the composite androgen response index is that the variation of the composite index is much smaller than using any individual androgen-responsive gene as readout, which will allow the use of a smaller sample size to achieve statistically significant results in future studies. Also, the composite index is more representative of the overall androgen response than any individual androgen-responsive genes.

We recognize that many factors could affect the expression of androgen-regulated genes without crosstalk with the AR in the prostate. However, AR remains the dominant factor regulating the expression of prostatic androgen-responsive genes. It is well established that the expression of androgen-responsive genes requires active AR and that their expression levels are dramatically down-regulated when the AR is inactive under castration conditions (19,29,30). Thus, the expression levels of androgen-responsive genes reflect AR activity in the prostate and, therefore, can serve as a reliable readout for androgen response.

It is well established that stromal-epithelial interaction plays a key role in androgen action in the prostate (31,32). However, the stromal androgen action is difficult to assess because very few stromal androgen-responsive genes have been reported. Androgen regulation of these potential stromal androgen-responsive genes *in vivo* is not clear. For example, Nemeth and colleagues showed that the expression of KGF *in vivo* is not greatly influenced by androgen manipulation (33). Without reliable readout for stromal androgen action, it is difficult to assess whether stromal androgen action is altered in BPH development.

Our results are in agreement with findings reported in the literature. Irer et al reported that androgen-responsive gene Nkx3.1 expression is increased in both mRNA and protein levels in BPH as compared to the non-BPH tissues (34). Also, ornithine decarboxylase activity and its mRNA expression are increased in BPH tissues (35) and ODC expression in the prostate is regulated by androgens (36). Our findings here and the data in the literature together strongly argue that androgen signaling is significantly elevated in BPH pathogenesis.

Alterations in androgen signaling in BPH appear to be very different from that in prostate cancer. Although androgens play important roles in the development and progression of both diseases, BPH tissues displayed a significant increase in the expression of androgen-responsive genes. In contrast, prostate cancer specimens exhibited down-regulation of androgen-responsive genes, including U19/Eaf2 (26), NKX3.1 (37), PMEPA1 (38) and many other androgen-responsive genes (39). Given that many of the androgen-responsive genes are tumor suppressive, down-regulation of tumor suppressive androgen-responsive genes may be an essential step leading to prostate cancer progression. In contrast, elevated expression of tumor suppressive androgen-responsive genes may be in part responsible for keeping BPH from becoming malignant. Difference in androgen-responsive gene expression between BPH and prostate cancer would represent a fundamental difference between these two common diseases.

The 4-fold up-regulation in the composite index of the expression of androgen-responsive genes in BPH is significant and likely to have functional impact on BPH pathogenesis. Thus, understanding the mechanism by which the androgen signaling is elevated in BPH glandular epithelial cells will have significant implications in BPH prevention and the treatment. One possible mechanism causing the elevated expression of androgen-responsive genes in BPH may involve inflammatory cytokines which are intimately associated with BPH and are known to enhance androgen signaling both in cultured cells and in animal models (40–50). Another possible mechanism may involve the enhanced expression of 5 α -reductase II (SRDA5) in BPH specimens (51). The elevated expression of SRDA5 may increase DHT levels in BPH areas relative to the normal adjacent tissues and lead to elevated expression of androgen-responsive genes. Both inflammation and elevated SRDA5 expression may contribute to the increased composite androgen response index in BPH cells. Clinical studies will be needed to determine the importance of inflammation, SRDA5, and other factors in the elevated expression of androgen-responsive genes in BPH.

In summary, our studies showed that the composite androgen-response index of 4 different androgen-responsive genes is elevated ~4-fold in BPH relative to adjacent normal tissues. This dramatic increase in androgen signaling is likely to play a key role in BPH development and progression. Elucidating the mechanisms responsible for the elevated androgen signaling in BPH may lead to new approaches of BPH prevention and treatment.

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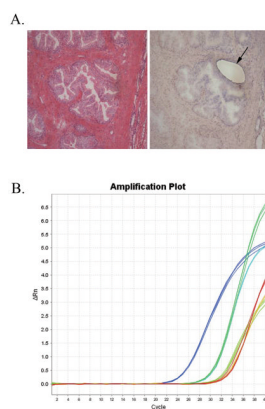
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**Fig 1.**

A. An H&E stained BPH (left) and its serial section after laser capture microdissection (LCM) (right). The arrow displays an area of a BPH epithelial nodule dissected for RT-qPCR. **B.** Representative real-time amplification of PSA (1), GAPDH (2), U19/EAF2 (3), and ELL2 (4) in LCM captured prostate epithelial cells. Epithelial cells were LCM dissected from fresh frozen human tissue.

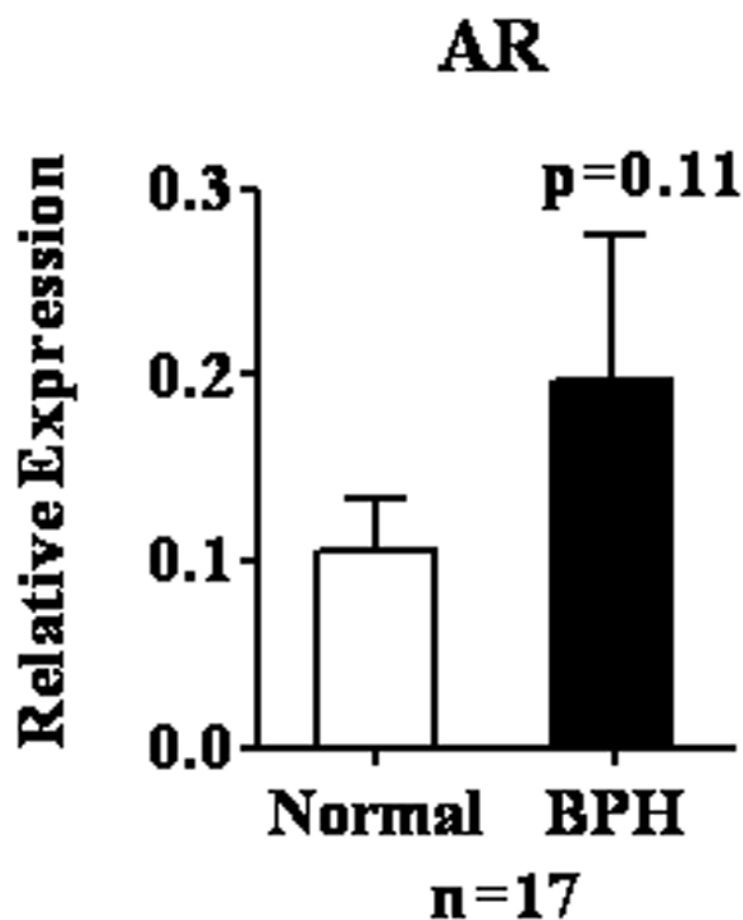


Fig 2.

Expression of androgen receptor (AR) in BPH and adjacent normal (Normal) glandular prostate epithelial cells. Prostate epithelia were laser-capture microdissected from fresh frozen prostate after briefly staining with H&E. RT-qPCR was performed and relative expression was analyzed as described in Material and Methods. The columns show the means; error bars represent S.E.M.; and n is number of specimens. The p values were calculated using the paired Student's t-test.

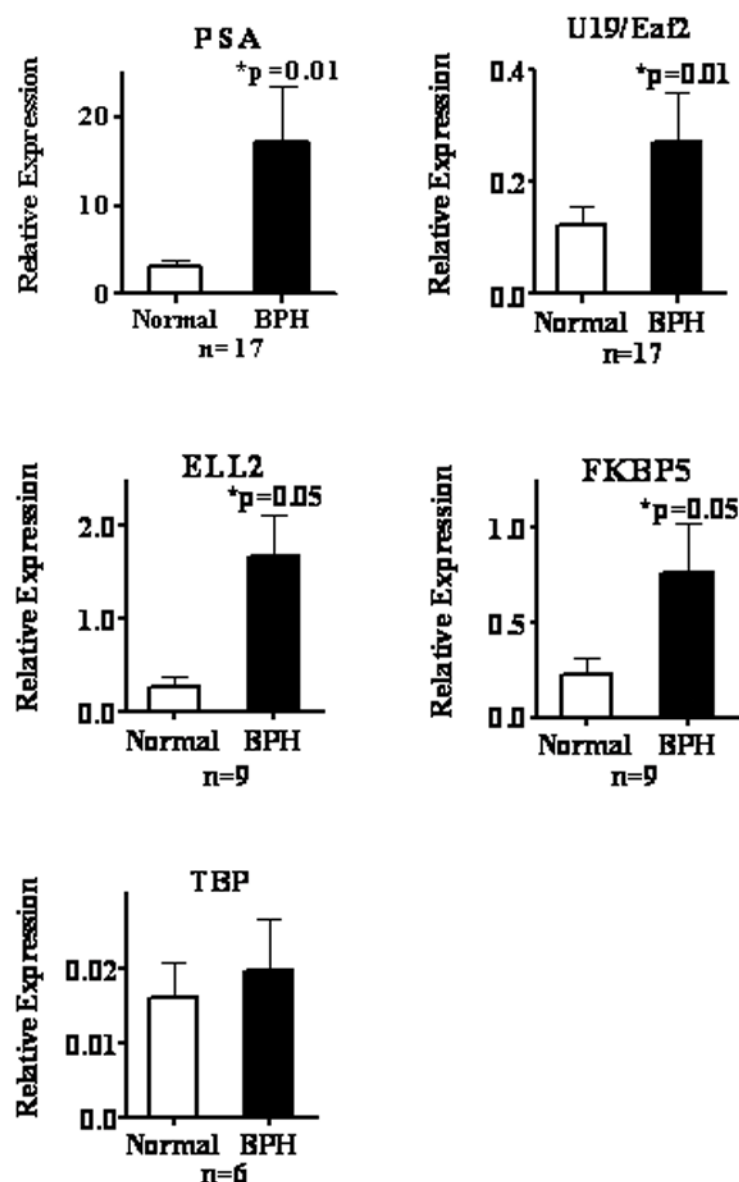
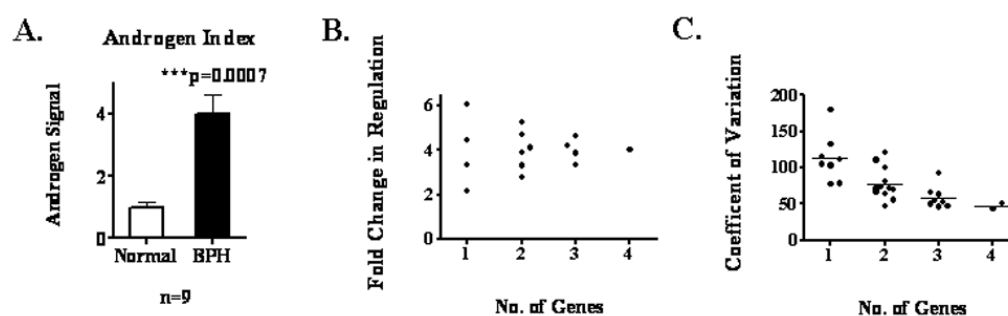


Fig. 3.

Expression of indicated genes in BPH and normal (Normal) areas adjacent to BPH in the transitional zone of human prostate. U19/Eaf2, ELL2, FKBP5, and PSA are androgen-responsive genes. TATA-box binding protein (TBP) is a housekeeping gene used as a control. The expression of each gene relative to GAPDH (Relative Expression) was determined as described in Material and Methods. The columns show the means; error bars represent S.E.M.; and n is number of specimens. The p values were calculated using the paired Student's t -test.

**Fig. 4.**

A. Composite androgen-response index (Androgen Signal; see text for definition) in BPH and adjacent normal (Normal) epithelial cells. The columns show the means; error bars represent S.E.M.; and n is number of specimens. The p value was calculated using the paired Student's t-test. **B.** Fold of androgen signaling up-regulation in BPH relative to adjacent normal areas. Four different androgen-responsive genes, ELL2 (6.0-fold), PSA (5.4-fold), FKBP5 (3.4-fold) and U19/Eaf2 (2.2-fold), were analyzed. The fold of up-regulation in composite androgen-response indexes was calculated using 1, 2, 3, or 4 genes in all possible combinations. **C.** Coefficient of Variation (%) of composite androgen-response indexes calculated using 1, 2, 3, or 4 genes in all possible combinations in both BPH and adjacent normal samples.

Table 1

Forward (For) and reverse (Rev) primers and corresponding probes in real-time PCR assays for indicated human gene expression in BPH specimens. TBP was purchased from ABI (Foster City, CA) and its sequence is proprietary and not included in the table. The specificity of the primer-probe combinations was confirmed by running parallel reactions with human genomic DNA, which did not amplify. The amplification efficiency of each primer pair was also indicated.

Gene	Primer/Probe	Efficiency
AR	For 5 -AAG CTG CAA GGT CTT CTT CAA-3 Rev 5 -TCC TTC GGA ATT TAT CAA TAG TGC-3 Probe 5 -6FAM AAC AGA AGT ACC TGT GCG CCA GCA TAMRA-3	99%
U19/Eaf2	For 5 -CCA GGA CTC CCA ATC TTG TAA A-3 Rev 5 -TAG CTT CTG CCT TCA GTT CTC TT-3 Probe 5 -6FAM CTC CAT CTG AAG ATA AGA TGT CCC CAG CA TAMRA-3	99%
ELL2	For 5 - TGA CTG CAT CCA GCA AAC AT-3 Rev 5 -TCG TTT GTT GCA CAC ACT GTA A-3 Probe 5 -6FAM TCT CCA GCT CTG GAG CCT CCC A TAMRA-3	92%
FKBP5	For 5 -AAA TCC AAA CGA AGG AGC AA-3 Rev 5 -GCC ACA TCT CTG CAG TCA AA-3 Probe 5 -6FAM AGT AGA AAT CCA CCT GGA AGG CCG CT TAMRA-3	99%
PSA	For 5 -CAT CAG GAA CAA AAG CGT GA-3 Rev 5 -AGC TGT GGC TGA CCT GAA AT-3 Probe 5 -6FAM CAC AGC CTG TTT CAT CCT GAA GAC ACA TAMRA-3	104%
GAPDH	For 5 -CAT GTT CGT CAT GGG TGT GA-3 Rev 5 -GGT GCT AAG CAG TTG GTG GT-3 Probe 5 -6FAM ACA GCC TCA AGA TCA TCA GCA ATG CCT C TAMRA-3	96%