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Original Article

# Quantitation of $\alpha_{1A}$ and $\alpha_{1D}$-adrenoceptor mRNA in prostate tissues from patients with symptomatic benign prostatic hyperplasia

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**Keywords:**

Adrenoceptor subtypes;
Benign prostatic hyperplasia;
Quantitative reverse transcription- polymerase chain reaction

## ABSTRACT

**Background:** To treat symptomatic benign prostatic hyperplasia $\alpha_1$-adrenoceptor antagonists with little antagonism at $\alpha_{1b}$-adrenoceptor were used to avoid orthostatic hypotension. In benign prostatic hyperplasia tissues $\alpha_{1D}$-adrenoceptor are thought to predominate, but in the Japanese experience, either $\alpha_{1A}$- or $\alpha_{1D}$-adrenoceptor antagonists can alleviate benign prostatic hyperplasia symptoms. We hypothesized that prostatic expression of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor varies quantitatively between patients.

**Materials and Methods:** We immunohistochemically localized $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor within benign prostatic hyperplasia tissues, and quantitated mRNA expression for these subtypes by real-time quantitative reverse transcription-polymerase chain reaction.

**Results:** Immunohistochemistry detected both subtypes in stromal but not detected epithelial cells. Copy numbers of $\alpha_{1A}$-adrenoceptor mRNA in benign prostatic hyperplasia tissue were significantly higher than those of $\alpha_{1D}$-adrenoceptor mRNA. Among patients; the ratio of $\alpha_{1A}$- to $\alpha_{1D}$-adrenoceptor mRNA ranged from 1.0 to 8.4.

**Conclusion:** An ideal therapeutic antagonist for treating benign prostatic hyperplasia symptoms should block both $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor

## INTRODUCTION

Since Price et al[1] have demonstrated three subtypes of α1-adrenoceptors (AR) in human prostatic tissue using molecular biologic techniques; several reports have described localization of these subtypes in prostatic tissue. Using semiquantitative immunohistochemical methods or radio-ligand binding assays, the subtype in prostatic tissue from patients with benign prostatic hyperplasia (BPH) has been found to be $\alpha_{1A}$.[1-3] Antagonists at $\alpha_1$-adrenoceptor (AR) have become the first-line therapy for BPH symptoms. Non-selective $\alpha_1$-AR antagonists such as prazosin, terazosin, and doxazosin were used first, and then superceded by a selective $\alpha_{1A}$-AR antagonist to avoid the common side effect of orthostatic hypotension.[4-6] The

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only $\alpha_{1A}$-AR selective antagonist previously available was tamsulosin, but a selective $\alpha_{1D}$-AR antagonist, naftopidil ((±)-1-[4-82-methoxyphenyl] piperazinyl)-3-(1-naphthlixy) propan-2-0l)], has been released.[7] In Japan, the only country where both agents are in common use for treating BPH; we sometimes encounter patients who respond poorly to tamsulosin but well to naftopidil, or vice versa.[8] These experiences led us to quantitate $\alpha_{1A}$- and $\alpha_{1D}$-AR expression in prostatic tissue from symptomatic BPH patients in order to test the hypothesis that expression of the subtypes varies between patients. Previous studies quantitating α1-AR subtypes in prostatic tissue have used a semi-quantitative RNase protection assay [1,3] or competitive reverse transcription- polymerase chain reaction (RT-PCR).[9] As real-time RT-PCR is the most sensitive and reproducible way to determine absolute numbers of specific mRNAs in samples, we adopted this method in addition to immunohistochemical analysis.

## MATERIALS AND METHODS

### Human prostatic tissue

Prostatic tissue specimens were obtained from 9 patients undergoing radical cystectomy to treat invasive bladder cancer at our institution or at affiliated hospitals. As these patients also had International Prostate Symptom Scores (I-PSS) of >17 and uroflowmetry results with a maximum flow rate of <10 ml/sec, they were all confirmed to have symptomatic BPH as well. Patients with nodules suggestive of prostate cancer and those with cancer invasion of the prostate or involvement of the trigone of the bladder were excluded, as were those with apparent urinary tract infection. Patients had no treatment with α1-AR antagonists for at least 1 month prior to the operation. None had a history of antiandrogen therapy or surgical intervention for BPH. All patients were fully informed about the study, and their prior consent was obtained.

Samples obtained from the resected prostate were processed for the procedures described below. Specimens were taken from the central area and the peripheral (subcapsular) area. The samples being used for RT-PCR were snap-frozen in liquid nitrogen and stored at -130°C and those for immunohistochemical staining were embedded in OCT compound and stored at -80°C until use.

### Antibodies and reagents for immunohistochemical staining

Goat antibodies raised against carboxy-terminal synthetic peptide fragments of human $\alpha_{1A}$-and $\alpha_{1D}$-ARs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). No cross-reaction was observed between antibodies as assessed using Rat-1 fibroblasts expressing the individual $\alpha_{1A}$-and $\alpha_{1D}$-ARs.[10] Avidin, biotin, and biotin-conjugated rabbit anti-goat antibody were purchased from Dako (Dako Japan, Tokyo, Japan). Streptavidin-conjugated horseradish peroxidase (HRP), 3-amino-9-ethylcarbazole (AEC) and crystal mount were purchased from Biomeda (Biomeda Co., Foster City, CA). Mayer's hematoxylin was purchased from Sigma (Sigma Chemical Co., St Louis, MO).

### Immunohistochemical staining

All steps were performed at room temperature unless otherwise indicated. Frozen sections were cut at a thickness of 4μm with a cryostat, fixed in acetone (10 min), and then hydrated in phosphate-buffered saline (PBS) (5-min washes). Nonspecific avidin- and biotin-binding sites were blocked by sequentially incubating with avidin for 10 min, washing with PBS, incubating with biotin for 10 min and washing with PBS. The sections were then incubated with 1% skim milk for 15 min and then the primary antibody (diluted 1:20 in skim milk) was applied overnight at 4°C. Sections were washed in PBS, then incubated with 1% hydrogen peroxide in methanol for 5 min to block endogenous peroxidase, washed again in PBS, and then incubated with a 1:400 dilution of biotin-conjugated rabbit anti-goat secondary antibody for 30 min. Then the sections were washed with PBS and incubated with streptavidin-conjugated HRP for 30 min. Following washing in PBS, sections were briefly rinsed with water and incubated with AEC. Color development was monitored by microscopy over 10 min. The color reaction was stopped by rinsing in water, after which sections were counterstained with 0.1% Mayer's hematoxylin solution for 3 min. Finally, the sections were rinsed under running water for 15 min and mounted in crystal mounting medium.

### Oligonucleotide primers and TaqMan probe for $\alpha_{1A}$- and $\alpha_{1D}$-AR

The primers and probes for the $\alpha_{1A}$- and $\alpha_{1D}$- ARs were determined with the assistance of the computer program Primers Express (Perklin-Elmer Applied Biosystems, Chiba, Japan), which selected theoretical optimized sequences for this system. Sequences of probe and amplification primer pairs as well as location of cDNA are listed in Table 1.

### Synthesis of recombinant RNA for $\alpha_{1A}$- and $\alpha_{1D}$-AR for generation of a standard curve

Partial-length wild-type cDNAs for $\alpha_{1A}$-AR and for $\alpha_{1D}$-

**Table 1. Primers and Probes**

| |
|---|
| Alpha 1A |
| Primer, forward; 5`-GGAATCTGTCTAGGAGCCCTCTCT-3 ( nt. 520-544) |
| Primer, reverse; 5-TCCCCAAGTTCTCCACTTACACA-3` ( nt. 580-603) |
| Probe; 5`-AAACTTGCCAACCTTCGTGTCAGGTGCT-3 ( NT. 550-578) |
| |
| Alpha 1D |
| Primer, forward; 5`-CCAGATGTCCACAACAATATGAAGT-3` (nt. 10-35) |
| Primer, reverse; 5`-TCCCCAAGTTCTCCACTTACACA-3` ( nt. 90-113) |
| Probe; 5`-CAGCAGAAGCCCCAAGCAACTTTC-3` (nt. 55-79) |

AR were subcloned in pGEM T-Easy vectors (Promega, Tokyo, Japan) and linearized with NcoI. Recombinant RNAs for $\alpha_{1A}$ - and $\alpha_{1D}$ -AR were generated by an in vitro transcription reaction with Sp6 RNA polymerase with the use of Riboprobe in vitro Transcription Systems (Promega), according to the manufacturer's instructions. Recombinant $\alpha_{1A}$- and $\alpha_{1D}$-AR RNA were quantified by spectrophotometry, divided into aliquots, and stored at –80°C until use.

**Extraction of RNA and TaqMan RT-PCR analysis**

After frozen tissue samples were homogenized in liquid nitrogen, total RNA was extracted from prostatic tissue with a commercially available kit (ISOGEN; Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Each RNA sample was quantified by its absorbance at 260 nm and stored at –80°C.

The reaction mixture (25 µl) containing 1X TaqMan Buffer A, 5.5 mM magnesium chloride, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 400 µM dUTP, 100 nM TaqMan probe, 400 nM primers, 0.625 U AmpliTaq Gold DNA Polymerase, 8 U RNase Inhibitor, 25 U MultiScribe Reverse Transcriptase, and 200 ng of DNase-treated total RNA was subjected to one-step RT-PCR.

Reaction conditions were 25°C for 10 min., 48°C for 30 min., and 95°C for 10 min. for 1 cycle of TaqMan RT reaction; followed by 40 cycles of polymerase reaction with 95°C for 15 sec and 60°C for 1 min. using an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems).

Each RT-PCR experiment included a standard curve assay with five known amounts of synthetic recombinant RNA (standard rRNA) in duplicate. For creating a standard curve for measurement of $\alpha_{1A}$-AR mRNA serial 10-fold dilutions of recombinant RNA ($10^{-8}$ to $10^{-12}$ g) and for measurement of $\alpha_{1D}$- AR mRNA serial 10-fold dilutions of recombinant RNA ($10^{-9}$ to $10^{-13}$ g) were used with blank assay without an RNA template. Subtraction of the baseline signal from the normal reporter signal was defined as ΔRn. This was plotted against the number of PCR cycles for each standard rRNA to determine the threshold PCR cycle (CT) where a significant increase in Rn above the baseline signal first could be detected. A standard curve for TaqMan real-time RT-PCR was created by plotting CT against initial template concentration.

**Determination of the mRNA levels of $\alpha_{1A}$- and $\alpha_{1D}$-AR**

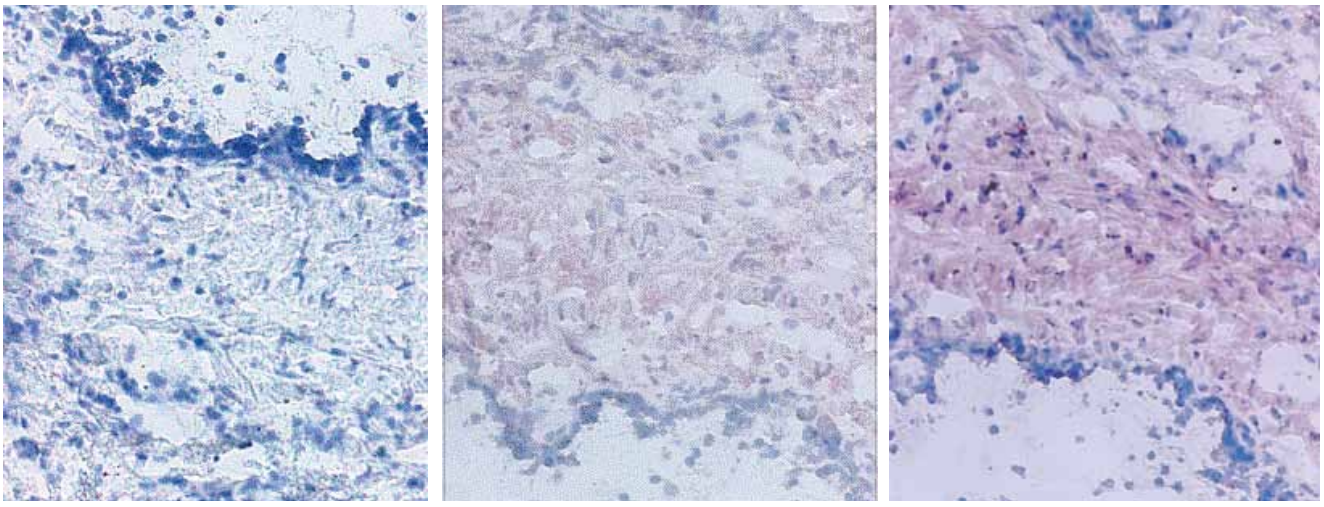
Copy numbers for standard curves of $\alpha_{1A}$- and $\alpha_{1D}$-AR RNA were calculated using the mean molecular weight of each recombinant RNA. Consulting the standard curve for each assay, the quantity of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA in the prostatic tissue specimen was determined as the copy number per microgram of total RNA extracted from each tissue sample.

**Statistical analysis**

Differences in expression of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA in prostate tissue were assessed by the Wilcoxon signed-rank test. Statistical analyses were performed using the StatView software package (SAS Institute, Cary, NC). P values < 0.05 were considered statistically significant.

## RESULTS

**Immunolocalization of α1A- and α1D-adrenoceptor in BPH tissue**



**Negative control** **Alpha 1A** **Alpha 1D**

(Original magnification, x100; bars indicate 100 µm)

***Figure 1:*** *Shows low-power surveys of the central zone of BPH specimens immunostained with specific goat anti-human $\alpha_{1A}$- and $\alpha_{1D}$-AR antibodies (panels A and B, respectively). The $\alpha_{1A}$-AR subtype was localized in the stromal component, and was not detected in epithelial cells. Similarly $\alpha_{1D}$-AR was detected only in stromal cells, predominantly smooth muscle cells, and not in epithelial cells.*

**Standard curve for quantification of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA by TaqMan real-time RT-PCR**

ΔRn increased during PCR as $\alpha_{1A}$-AR PCR copy number increased until the reaction reached a plateau at 40 cycles of PCR. A representative result is shown in fig. 2A.

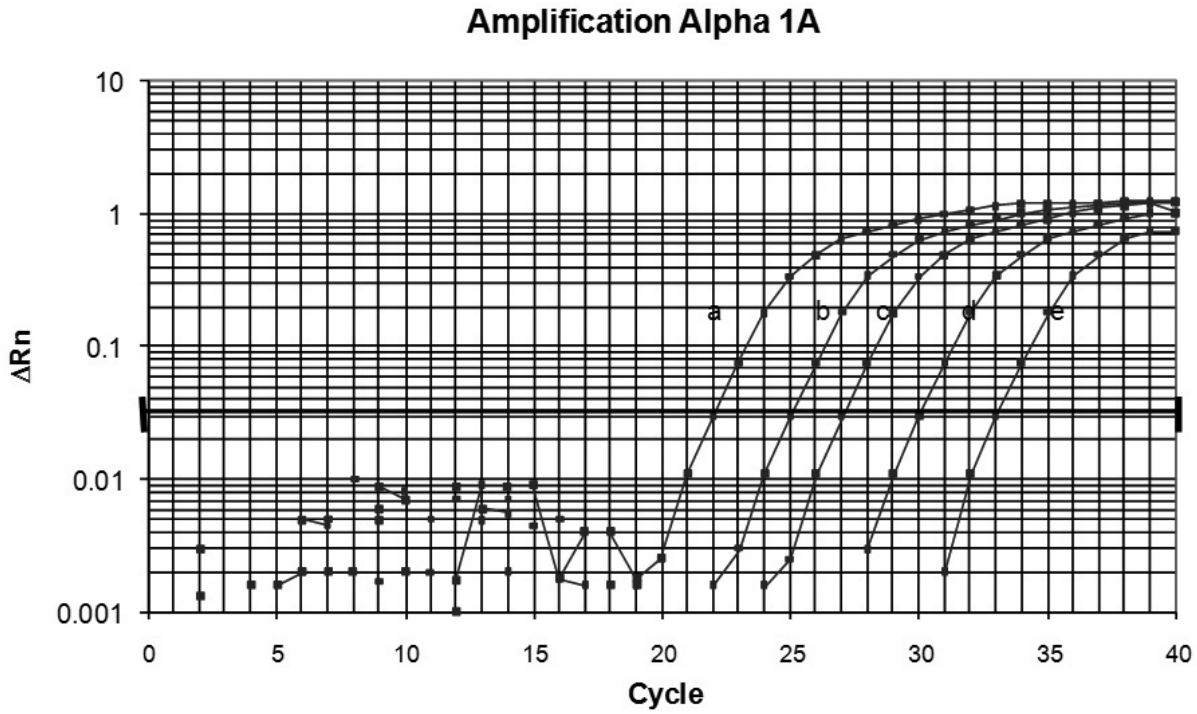



***Figure 2A:*** *An $\alpha_{1A}$-adrenoceptors (AR) standard curve for TaqMan real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).*

Amplification plots for reactions showing five points (a, b, c, d, and e) on the $\alpha_{1A}$-AR standard curve (serial 10-fold dilutions of recombinant RNA for $\alpha_{1A}$-AR). ΔRn represents the normalized reporter signal (Rn) minus the baseline signal. ΔRn increases during PCR as $\alpha_{1A}$-AR PCR product copy number increases until the reaction reaches a plateau.

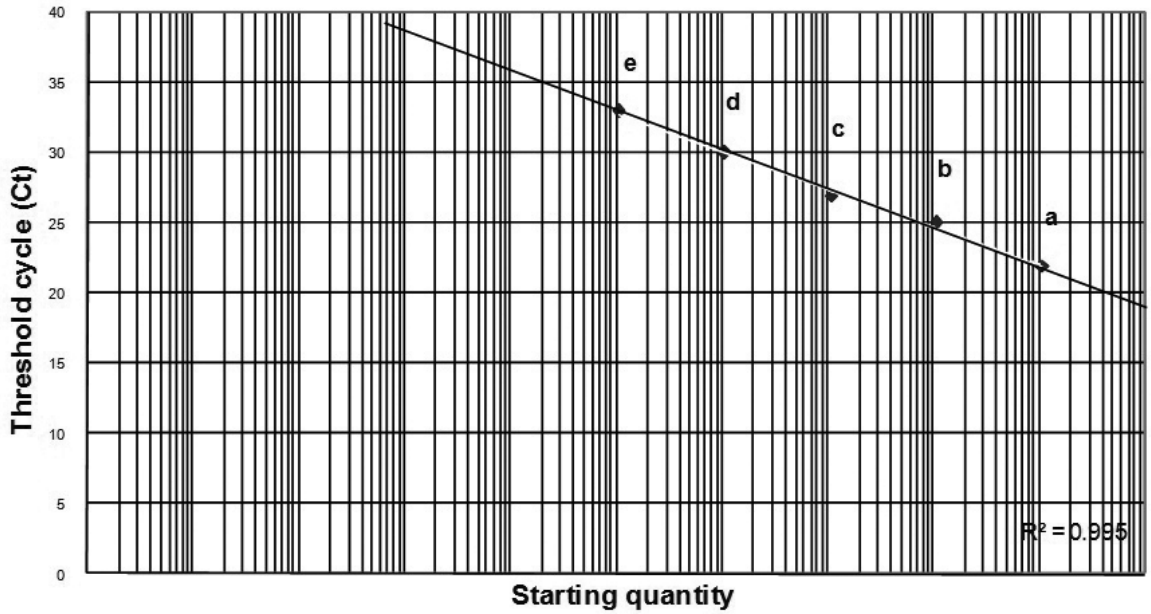



***Figure 2B:*** *A standard curve plotting logarithm of the starting copy number vs. threshold cycle (CT) to determine the initial template concentration. CT represents the PCR cycle at which a significant increase in Rn above a baseline signal first can be detected.*

Threshold cycle (CT) was plotted against logarithms representing the serial 10-fold dilutions of synthetic RNA to plot a standard curve for determination of the amount of $\alpha_{1A}$-AR mRNA (fig. 2B).

For both $\alpha_{1A}$- and $\alpha_{1D}$-AR RNA a strong linear relationship was always shown between the threshold cycle (CT) and the logarithm of the starting RNA copy number; $R^2$ always exceeded 0.99.

**Quantitation of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA by TaqMan real-time RT-PCT in the prostate tissue from patients with BPH**

Expression of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA in prostate tissue from symptomatic BPH patients was quantitated by real-time RT-PCR. In central and peripheral areas of the prostate, respectively, 3.76-37.2x$10^7$ and 5.4-68.1x$10^7$ copy numbers of $\alpha_{1A}$-AR mRNA per microgram of total RNA were detected. Copy numbers of $\alpha_{1D}$-AR mRNA were 0.46-29.2x$10^7$ in the central area and 0.64-58x$10^7$ in the peripheral area (fig. 3A, B).

No statistically significant difference in the amount of $\alpha_{1A}$- or $\alpha_{1D}$-AR mRNA was found between the central and peripheral areas of the prostate. The amount of $\alpha_{1A}$-AR mRNA was consistently higher than that of $\alpha_{1D}$-AR mRNA regardless of location in the prostate. However, ratios of $\alpha_{1A}$- to $\alpha_{1D}$-AR mRNA expression were distributed in a wide range in both areas (1.0 to 8.4; fig. 3C).

Fig. 3A

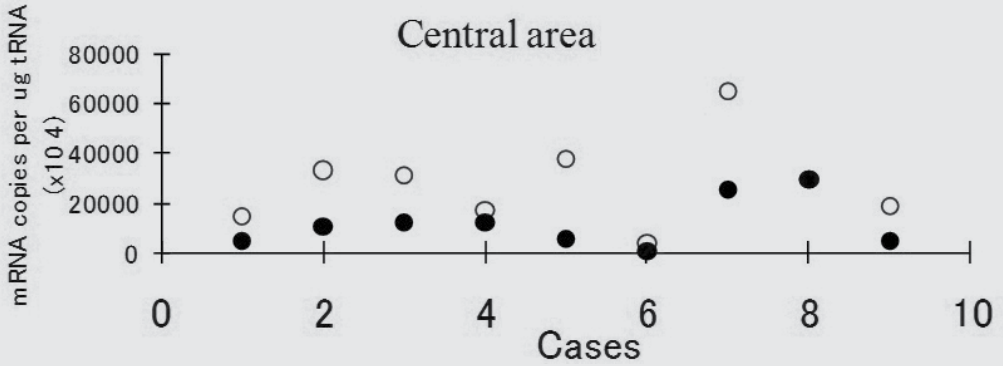



Fig. 3B

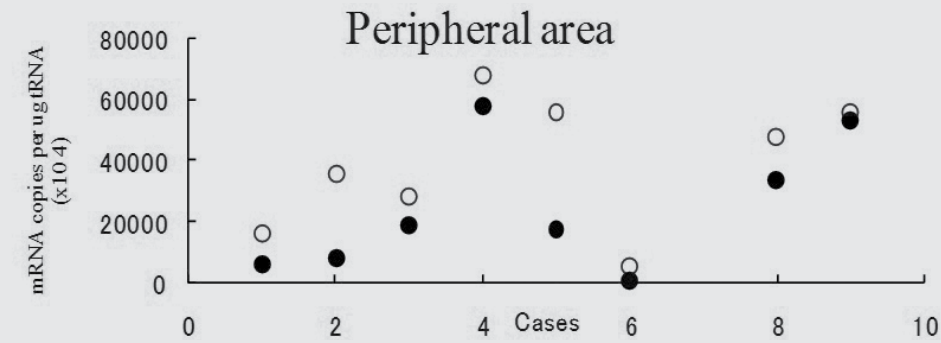



Fig. 3C

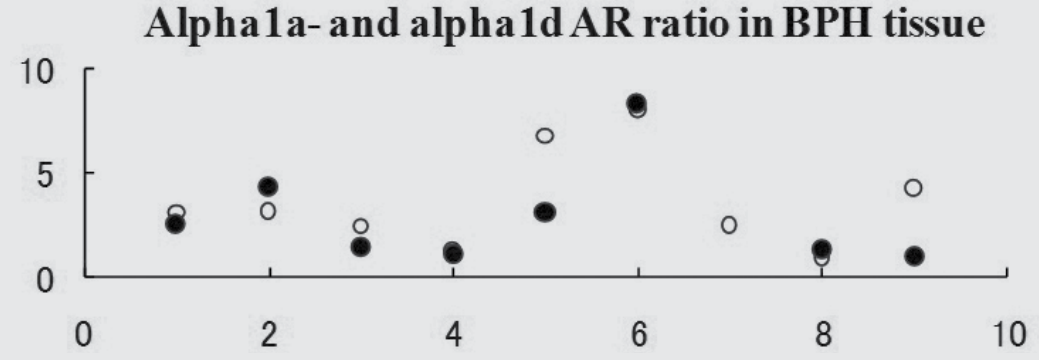



***Figure 3:*** *Amounts of $\alpha_{1A}$- and $\alpha_{1D}$-adrenoceptor (AR) mRNA in central and peripheral areas of the hyperplastic prostate.*

***A:*** *Amounts of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA in the central area of the prostate.*

***B:*** *Amounts of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA in the peripheral area of the prostate.*

*In A and B, open circles indicate $\alpha_{1A}$-AR mRNA; closed circles indicate $\alpha_{1D}$-AR mRNA.*

***C:*** *Ratio of $\alpha_{1A}$-AR mRNA to $\alpha_{1D}$-AR mRNA in central (open circles) and peripheral (closed circles) areas of the prostate.*

## DISCUSSION

Various studies have demonstrated that α1-AR in the prostate, bladder neck, and urethra are responsible for regulating smooth muscle tone, which thereby causes the dynamic component of obstruction in lower urinary tract syndromes (LUTS) suggestive of BPH. [1,2,10-12] Localization of mRNA for α1-AR subtypes has been investigated with nucleic acid probes and localization of the receptor protein was assessed by subtype-specific ligands.[7, 13, 14] However, expression and localization of receptor mRNA may not accurately reflect receptor protein levels. Lack of an appropriate probe for receptor protein has hampered determination of expression of receptor proteins at the cellular level. Recently, a group of antibodies raised to carboxy-terminal synthetic peptide fragments of human $\alpha_{1A}$- and $\alpha_{1D}$- AR became commercially available.[15] We used these specific antibodies for immunolocalization of $\alpha_{1A}$- and $\alpha_{1D}$-AR proteins in BPH tissue.

Both $\alpha_{1A}$-AR and $\alpha_{1D}$-AR showed immunoreactivity in the stromal tissue of the prostate, and no immunostaining was seen in the epithelial tissue. Our results confirmed those of previous reports [1,3] except for those of Walden, et al [16], who detected abundant $\alpha_{1D}$-AR in areas of stromal tissue where smooth muscle cells predominated, but also found abundant $\alpha_{1D}$-AR in blood vessels. Although the intensity of $\alpha_{1A}$-AR immunostaining appeared stronger than that of $\alpha_{1D}$-AR, our finding was nonquantitative and subjective. We therefore quantitatively assayed mRNA expression for these subtypes of α1-AR.

Characterization of α1-AR subtypes in the human prostate initially relied upon pharmacologic methods. Following the introduction of molecular techniques, expression of mRNA for the three subtypes has been examined by RNase protection assays and in situ hybridization[1,9] However, few reports have provided truly quantitative analyses of receptor subtype expression in BPH tissue. In this study we used TaqMan real-time RT-PCR analysis for quantitation of mRNA for two α1-ARs. The theoretical basis of the measurement can be described briefly as follows.

The TaqMan assay utilizes the 5'→3' exonuclease activity of Taq DNA polymerase and a fluorogenic probe for automated quantification of DNA in real time. The CT value refers to the threshold cycle at which a statistically significant increase in fluorescence is first detected by the sequence detection system. The increase in fluorescence is directly proportional to the exponential increase in PCR product, and signal measurement is carried out in a real-time manner. Therefore, samples containing few target molecules would require more PCR cycles (hence higher CT) to amplify enough copies to produce significant fluorescent signal.

Using this TaqMan system, we succeeded in determining amounts of mRNA for $\alpha_{1A}$-adrenoceptor subtypes in a highly reproducible manner. This system showed a very small coefficient of variation (CV) in the preliminary experiments, with inter-assay and intra-assay CV consistently less than 1% (data not shown). We therefore adopted this precise method to quantitate mRNA for adrenoceptor subtypes in BPH tissue. We believe that this is the first study to use this technique to evaluate prostatic α1-AR mRNA expression.

Expression of $\alpha_{1A}$-AR mRNA was consistently higher than that of $\alpha_{1D}$-AR mRNA in both centrally and peripherally located prostate tissue specimens from patients with BPH. No statistically significant difference was noted in $\alpha_{1A}$-AR mRNA content between central and peripheral areas, as in previous reports.[9] Expression of $\alpha_{1D}$-AR mRNA also did not differ between central or peripheral areas of the hyperplastic prostate. There is no previous data available regarding the regional differences in mRNA for $\alpha_{1D}$-AR in BPH tissue.

Finally, we calculated ratios of $\alpha_{1A}$- to $\alpha_{1D}$-AR mRNA in central and peripheral areas of the prostate from patients with BPH. This ratio did not show a significant difference between central and peripheral areas. In previous reports the ratio ranged between 3.8 and 10.1 3, while our ratios ranged between 1.0 and 8.4.

For medical treatment of BPH symptoms, the first-line therapeutic agent is an α1-AR antagonist. Several α1-AR antagonists are used in various countries. To reduce the incidence of orthostatic hypotension, which is common with nonselective α1-AR antagonists, subtype-selective α1-AR antagonists now are favored. Orthostatic hypotension is associated with α1B-AR antagonism. After introduction of tamsulosin, which is fairly selective for $\alpha_{1A}$- AR compared with $\alpha_{1D}$-AR, several agents that are still more selective or super selective for $\alpha_{1A}$-AR have been developed and are going under clinical trials. Our data indicated that ratios of $\alpha_{1A}$- to $\alpha_{1D}$-AR expression differs between patients. Some patients can be treated successfully with α1-AR antagonists specific for $\alpha_{1A}$-AR, but others may have better results using antagonists with at least some activity at $\alpha_{1D}$-AR.

## CONCLUSION

An ideal therapeutic agent for BPH symptoms would not be an antagonist super selective for $\alpha_{1A}$-AR, but would block both $\alpha_{1A}$- and $\alpha_{1D}$-AR to some extent.

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