Evaluation of Androgen, Estrogen (ERα and ERβ), and Progestrone Receptor Expression in Human Prostate Cancer by Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction Assays

Alain Latil,1 Ivan Bièche, Dominique Vidaud, Rosette Lideureau, Philippe Berthon, Olivier Cussenot, and Michel Vidaud

ABSTRACT

Steroid hormones can have profound effects on prostate tumor development making it important to define steroid receptor expression in prostate tissues. For this purpose, androgen receptor (AR) and estrogen receptor (ERα and ERβ) expression was quantified in 12 clinically localized and 11 hormone-refractory sporadic prostate tumors, using real-time quantitative reverse transcription-PCR assays. To gain more insight into hormone-responsiveness, estrogen-regulated progesterone receptor (PGR) and androgen-regulated prostatic acid phosphatase (PAP) mRNA levels were also quantified. There is a decrease in expression of ERβ in both clinically localized and hormone-refractory tumors relative to normal prostate tissues. Moreover, hormone-refractory tumors display a decreased expression of ERα and an increased expression of AR. There is a positive association between ERα, ERβ, and PGR expression (P < 0.0001) and a negative association between AR and the androgen-regulated gene PAP expression in hormone-refractory tumors. Taken together, these data indicate that, although increased expression of the AR gene might play a key role in endocrine treatment failure, it cannot be considered the sole actor of this unresolved dilemma, and abnormalities in ERα and/or ERβ expression may also modulate the growth response of prostate cancer to hormone withdrawal. Our results also suggest that ERα and ERβ expression status could be used to identify advanced prostate tumor patients who may respond to antiestrogen therapy.

INTRODUCTION

CaP2 is one of the most common forms of cancer in men in developed countries (1), and the frequency of mortality is expected to increase unless improved treatments can be found. CaP is initially dependent on androgen stimulation mediated by the AR a member of the steroid hormone receptors family (2). The receptor functions as a ligand-induced factor, promoting transcriptional activation (or repression) of its target genes (3). Increased expression of the AR gene may play a key role in failure of conventional androgen-deprivation therapy in advanced CaP (4).

If the importance of AR in the evolution of CaP is well established, many reports also deal with the potential implication of two other members of the steroid hormone receptor family, ERα and PGR, in prostatic carcinogenesis (5–8). ERα is an estrogen-dependent transcriptional factor that regulates growth, development, differentiation, and homeostasis by binding to EREs in DNA to modulate transcription of target genes (9), including PGR. There has never been a clear demonstration of direct estrogenic effects through a receptor-mediated process in human prostate tumors. However, stimulatory or inhibitory effects of estrogen on in vitro growth of CaP cells (10), along with the demonstration of the presence of ERα in CaP cell lines, normal, and cancerous prostate tissues (5–8), suggest that this steroid hormone may exert direct effects on prostate via its own receptor. In the normal human prostate, immunohistochemical and ISH studies have revealed a stromal localization of ERα and PGR expression. Little or no ERα expression was detected in malignant prostatic epithelium, whereas it has been reported in several CaP cell lines including LNCaP, DU145, PC3 (10, 11), although not confirmed (12). Regardless of these divergent findings on ERα expression, the identification of a gene encoding a second type of ER, termed ERβ, has shed new light on the role of estrogens in prostate tissue (13). ERβ is expressed at high levels in epithelial compartments of the rat and monkey prostate gland (13–15). Despite a recent report that noted expression of ERβ in basal epithelial cells (16), knowledge of the distribution of this receptor in normal and neoplastic human prostate tissues is limited. Although little or no expression of PGR, the major ERα responsive gene, was detected in malignant prostatic epithelium, positive staining of PGR protein has been observed by radioligand binding assays, and immunohistochemical analysis (8). Moreover, a positive stain for PGR was revealed in the LNCaP CaP cell line (10).

A first step in understanding steroid action on CaP is to define receptor expression in prostate tissues. Although there have been many reports dealing with steroid hormone receptors in prostate tissues, their expression has yet to be studied simultaneously and by an accurate quantitative method.

To elucidate the respective contributions of steroid hormone receptor genes and their interaction in CaP, AR, ERα, ERβ, and PGR expression were quantified in 23 sporadic prostate tumors using real-time quantitative RT-PCR assays. This method of nucleic acid quantification in homogeneous solution is a reference in terms of performance, accuracy, sensitivity, wide dynamic range and high throughput; it eliminates the need for tedious post-PCR processing. To gain more insight into hormone-responsiveness, in addition to the estrogen-regulated PGR gene, mRNA levels of androgen-regulated PAP were also quantified.

MATERIALS AND METHODS

Patients and Samples

Twenty-three primary prostate tumors obtained from patients undergoing surgery at St. Louis Hospital in Paris, La Cavale Blanche Hospital in Brest, and the Centre Hospitalier Universitaire in Nancy (France) were analyzed. Twelve patients had clinically localized prostate tumors and 11 had hormone-refractory recurrent prostate carcinomas.

Prostate samples were obtained in two ways as follows: (a) clinically localized prostate tumors were obtained by radical prostatectomy. Specimens were first sliced thickly, and samples were cut from suspect areas. Part of the selected tissue was immediately placed in liquid nitrogen for RNA extraction,
and adjacent sections were stained with H&E to determine the proportion of tumor cells in each sample. After pathological examination, 7 of the 12 selected clinically localized tumors were limited to the prostate (pT1N0M0), whereas local extracapsular extension (without regional lymph node involvement) was observed in 5 (pT2N0M0); and (b) patients with metastatic disease, for which radical surgery is excluded, have been treated by androgen deprivation (by removal of the testes, by antiandrogen). After varying times, these patients relapse and their tumors become clinically androgen independent. These hormone-refractory recurrent prostate carcinomas were obtained by transurethral resection and 6–12 chips were obtained during resection.

Selected Tissues

Malignant areas from tumor samples were carefully selected by means of microdissection to obtain a homogeneous cell population and thereby avoid “dilution” of tumor-specific genetic changes with RNA from normal and reactive cells present in the same specimen. For these reasons, a sample was considered suitable for molecular studies if the proportion of tumor cells exceeded 90% of epithelial cells.

The histological diagnosis, clinical staging based on the TNM system and Gleason score (17, 18) were determined in each case during a routine clinical work-up after surgery.

Four well-characterized normal prostate tissue specimens were used to assess basal levels of mRNA from target genes in normal prostate tissue.

Normal prostate tissues were obtained from 4 of the 20 patients who underwent a radical prostatectomy. Samples were histologically checked and selected for the absence of cancer cells and in respect to their cell type component; indeed, to avoid benign prostatic hyperplasia, samples with a majority of stroma cells were excluded from this study.

Real-Time RT-PCR

Theoretical Basis. Quantitative values are obtained from the threshold cycle (Ct) number at which the increase in signal associated with an exponential growth of PCR product starts to be detected (using Perkin-Elmer Biosystems analysis software), according to the manufacturer’s manual.

The precise amount of total RNA added to each reaction (based on absorbance) and its quality (i.e., lack of extensive degradation) are both difficult to assess. We, therefore, also quantified transcripts of the gene RPLP0 (also known as 36B4) encoding human acidic ribosomal phosphoprotein P0 as the endogenous RNA control, and each sample was normalized on the basis of its RPLP0 content. The relative target gene expression level was also normalized to a calibrator (pool of four normal human prostate tissue specimens).

Final results, expressed as N-fold differences in target gene expression relative to the RPLP0 gene and the calibrator, termed “Ntarget,” were determined as follows:

\[
N_{\text{target}} = 2^{\Delta C_{\text{target}} - \Delta C_{\text{calibrator}}}
\]

where \(\Delta C\) values of the sample and calibrator are determined by subtracting the average Ct value of the target gene from the average Ct value of the RPLP0. Because of the problem of prostate cellularity and of the cell type specificity expression of the genes of interest, quantification was assessed according to the epithelial and stromal cell component of each sample. For this purpose, transcripts of cytokeratin 18 (KRT18), a specific epithelial marker, and PGR in human prostate tissues

The expression levels are displayed as a ratio between the target gene and a reference gene (RPLP0) to correct for variation in the amounts of RNA as well as of that specific marker for epithelial cells (KRT18) and/or stroma cells (VIM) according to the cell-type specificity expression of the target gene. RPLP0, KRT18, and VIM denote acidic ribosomal phosphoprotein P0, cytokeratin 18, and vimentin.

Table 2. Quantification of AR, ERα, ERβ, and PGR in human prostate tissues

<table>
<thead>
<tr>
<th>Samples</th>
<th>Normala (mean ± SD)</th>
<th>Clinically localized (mean ± SD)</th>
<th>Hormone-refractoryb (mean ± SD)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR-RPLP0</td>
<td>1.00 ± 0.15</td>
<td>1.15 ± 0.54</td>
<td>7.38 ± 7.23</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AR-KRT18</td>
<td>1.00 ± 0.15</td>
<td>1.00 ± 0.38</td>
<td>12.31 ± 13.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ERα-RPLP0</td>
<td>1.00 ± 0.20</td>
<td>0.71 ± 0.52</td>
<td>0.58 ± 0.54</td>
<td>NSb (P = 0.16)</td>
</tr>
<tr>
<td>ERα-VIM</td>
<td>1.00 ± 0.38</td>
<td>1.29 ± 0.65</td>
<td>0.59 ± 0.45</td>
<td>0.02</td>
</tr>
<tr>
<td>ERβ-RPLP0</td>
<td>1.00 ± 0.20</td>
<td>0.47 ± 0.46</td>
<td>0.38 ± 0.45</td>
<td>0.02</td>
</tr>
<tr>
<td>ERβ-KRT18</td>
<td>1.00 ± 0.31</td>
<td>0.38 ± 0.39</td>
<td>0.62 ± 0.64</td>
<td>NS (P = 0.07)</td>
</tr>
<tr>
<td>ERβ-VIM</td>
<td>1.00 ± 0.23</td>
<td>1.16 ± 1.00</td>
<td>0.52 ± 0.48</td>
<td>NS (P = 0.23)</td>
</tr>
<tr>
<td>PGR-RPLP0</td>
<td>1.00 ± 0.74</td>
<td>0.42 ± 0.38</td>
<td>1.30 ± 1.25</td>
<td>NS (P = 0.18)</td>
</tr>
<tr>
<td>PGR-VIM</td>
<td>1.00 ± 1.00</td>
<td>0.90 ± 0.65</td>
<td>1.90 ± 2.00</td>
<td>NS (P = 0.67)</td>
</tr>
</tbody>
</table>

* The ratios for each type of analysis have been normalized in such a manner that the mean ratio of the normal prostate samples equals a value of 1.
* The values for T101 were excluded from the calculation because they are inconsistent with those for the hormone refractory tumors.
* Ps were calculated by the Kruscal-Wallis test or the Mann-Whitney U test.
* NS, not significant.
and of vimentin (VIM), a specific mesenchymal marker, were also quantified and used as specific endogenous controls.

Each sample was, thus, also normalized on the basis of its KRT18 and/or VIM content.

Primers and PCR Consumables. Primers were chosen with the assistance of the computer programs Oligo 4.0 (National Biosciences, Plymouth, MN) and Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA). We performed BLASTN (19) searches against dbEST and nr (the nonredundant set of GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen as primers. To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or in a different exon. Primer sets were, furthermore, checked on PCR reaction for a single band on agarose gel, and their products were purified and sequenced to confirm the specificity.

The nucleotide sequences of primers are shown in Table 1.

RNA Extraction. Total RNA was extracted from tissue specimens by using the acid-phenol guanidium method (20). The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S RNA bands were visualized under UV light.

cDNA Synthesis. cDNA was reverse transcribed in a final volume of 20 μl containing 1× reverse transcriptase buffer [500 mm each dNTP, 3 mm MgCl2, 75 mm KCl, and 50 mm Tris-HCl (pH 8.3)], 10 units of Rnasin RNase inhibitor (Promega, Madison, WI), 10 μM dTT, 50 units of Superscript II RNase H reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD), 1.5 mm random hexamers (Pharmacia, Uppsala, Sweden), and 1 μg total RNA. The samples were incubated at 20°C for 10 min and at 42°C for 30 min, and reverse transcriptase was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

PCR Amplification. All of the PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). PCR was performed using the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and 65°C for 1 min. Specific PCR amplification products were detected by the fluorescent double-stranded DNA-binding dye, SYBR Green (21). Experiments were performed with duplicates for each data point. All of the samples with a coefficient of variation for Ct value higher than 1% were retested.

Statistical Analysis

The nonparametric Mann-Whitney U and Kruskal-Wallis tests (StatView 1996, Abacus Concepts, Inc., Berkeley, CA) were used to evaluate the variation in expression between prostate tissues. To analyze the correlation of expression levels between two different genes, regression analysis was used.

RESULTS

The relative expression levels of hormone steroid receptors were quantified in 23 tumors (T) and 4 normal prostate tissues (N). The expression levels are determined as ratios between AR, ERα, ERβ, PGR, PAP and the reference gene RPLP0 to correct for variation in the amounts of RNA. Because prostate samples contained mixtures of cell types, KRT18 and/or VIM, according to the target gene cell-type specificity, were also used as reference to quantify the expression levels of AR, ERα, PGR, and PAP. Because of ERβ expression's...
unknown cell type specificity, both KRT18 and VIM were used as reference genes for its expression. Results are summarized in Table 2 and illustrated in Figs. 1 through 5.

**AR Expression in Prostate Tissues.** The mean expression levels of AR in localized prostate tumors was similar to that in normal prostate tissues, whereas it was significantly higher in hormone-independent tumors, when RPLP0 was used for normalization (P < 0.001; Table 2; Fig. 1A). Hormone-refractory tumors displayed increased expression of AR, varying from 2- to 40-fold higher than normal prostate tissues (Fig. 1, A and B).

**ERα Expression in Prostate Tissues.** When RPLP0 was used for normalization, a trend toward ERα down-regulation was observed in tumors compared with normal prostate tissues (P = 0.02; Table 2); indeed, ERα expression is reduced in 10 of 12 clinically localized prostate tumors and 9 of 11 hormone-refractory tumors (Fig. 3A). Although not significant (Table 2), a trend toward ERα down-regulation was confirmed in most of prostate tumors when KRT18 was used for normalization (Fig. 3B).

Similar results in ERβ mRNA levels were observed when VIM was used for normalization (Fig. 3C). Irrespective of the reference gene used, one hormone-refractory tumor (T101) showed very high ERβ mRNA levels (Fig. 3).

**ERβ Expression in Prostate Tissues.** ERβ mRNA levels are significantly lower in prostate tumors than in normal prostate tissues when RPLP0 was used for normalization (P = 0.02; Table 2); indeed, ERβ expression is reduced in 10 of 12 clinically localized prostate tumors and 9 of 11 hormone-refractory tumors (Fig. 3A). Although not significant (Table 2), a trend toward ERβ down-regulation was confirmed in most of prostate tumors when KRT18 was used for normalization (Fig. 3B).

**PGR Expression in Prostate Tissues.** There is no significant association between PGR expression and prostate tissues (Table 2). However, the overall trend is toward a decreased expression in clinically localized tumors and an increased PGR expression in hormone-refractory tumors compared with normal prostate tissues when RPLP0 was used for normalization (Fig. 4A). When VIM was used as an endogenous control, an apparent decrease in expression of PGR was observed in four clinically localized tumors and four hormone-refractory tumors, whereas three hormone-refractory tumors (T60, T101, T105) showed more than a 3-fold increase in expression (Fig. 4B).

**PAP Expression in Prostate Tissues.** Comparison of PAP expression between normal prostate and tumor tissues revealed similar or
increased expression in clinically localized tumor specimens (except T22 and T108), irrespective of the reference gene used for normalization (RPLP0 or KRT18). Most of the hormone-refractory tumors showed a decreased expression of PAP (Fig. 5).

**Relationships of mRNA Expression Levels among AR, ERα, ERβ, PGR, and PAP.** There is a positive association between ERα and ERβ expression regardless of which reference gene is used (P < 0.0001; Fig. 6); tumors that showed low levels of ERα also showed low levels of ERβ, whereas T101 showed the highest expression in both ERα and ERβ.

There is a positive association between PGR and ERα expression (P = 0.0039 and P < 0.0001, Fig. 7, A and B, respectively) and between PGR and ERβ expression in prostate tissues (P = 0.0019, and P < 0.0001; Fig. 7, C and D, respectively) regardless of which reference gene is used.

PAP expression is inversely related to AR expression in hormone-refractory tumors (data not shown).

There is no significant association between AR and ERα or ERβ expression.

**DISCUSSION**

To gain insight into the role of the steroid hormone receptors in human prostate adenocarcinomas, we quantified AR, ERα, ERβ, PGR, and PAP mRNA expression levels in 23 sporadic prostate tumors (12 clinically localized and 11 hormone-refractory recurrent prostate carcinomas) using quantitative real-time RT-PCR assays.

To take into account the variability of the quantity of RNA introduced into the reaction, the expression of the genes of interest was determined using RPLP0 as an endogenous RNA control. Because the relative determinations of expression level can be affected by cellular composition of the tissue sample, KRT18, an endogenous marker of epithelial cells and/or VIM, a stroma-specific control, were also used as reference genes. In each sample, expression levels of KRT18 and VIM, by means of quantitative real-time RT-PCR, was correlated with histomorphological analysis of the corresponding tissue section (data not shown).

AR expression was similar in clinically localized prostate tumor and normal prostate tissues, whereas most of the hormone-refractory tumors showed significantly higher AR mRNA levels (P < 0.0001, Table 2); indeed AR expression level in hormone-refractory tumors ranged from 2.0- to 39.0-fold higher than in the normal prostate tissues when KRT18 was used as reference (Fig. 1B). These results are consistent with previous reports on AR expression in hormone-refractory prostate tumors (22, 23).
As a matter of fact, patients with metastatic disease at diagnosis, for which radical surgery is excluded, have been treated by endocrine therapy, either by classical androgen deprivation (orchitectomy or LHRH agonist) or by maximal androgen blockade (castration combined with antiandrogen). When these patients relapse, their tumors are clinically androgen independent; all of the 11 tumors that are androgen refractory show high AR levels, which suggests that the AR gene may play a role in endocrine treatment failure. However, the AR is not activated conventionally, because mRNA levels of PAP, a gene normally induced through its ARE, remain low after androgen withdrawal. Thus, either AR is not functional in hormone-refractory tumors but up-regulated in an absence-of-ligand-dependent manner (i.e., the nonactivation of the AR by its ligand triggers its own overexpression) or AR overexpression is involved in androgen-independence by a different AR-mediated mechanism. As a matter of fact, because there is no positive association between AR and PAP expression, if AR overexpression is truly involved in hormone independence of prostate tumors, it can be hypothesized that mutant AR could enhance target gene activity via responsive elements other than AREs, including EREs (several hormone-refractory tumors show high PGR expression levels), cAMP responsive elements, and/or, in a somewhat different manner, by coupling growth factors to multiple signaling pathways that converge in a tissue-specific response (24).

It is noteworthy that there is no correlation between AR expression level and the treatment (LHRH agonist or antiandrogen).

If the importance of AR in human CaP has been established, no conclusive evidence exists at present regarding the role of ER in CaP. We detected ERO and ERβ subtypes in both normal and neoplastic prostate tissues. The mean expression levels of ERO appeared to be down-regulated in many tumors when RPLP0 was used for normalization (Fig. 2B). In clinically localized tumors, this apparent down-regulation was absent when the specific-stroma cell gene, VIM, was used for normalization. These results indicate that either ERO is truly down-regulated in clinically localized tumors and otherwise normally expressed in a component cell-independent fashion or that the ERO gene expression is stroma dependent, which accounts for its apparent down-regulation in tumors using RPLP0 as reference gene. The latter scenario seems more likely in light of previous reports using ISH and IHC to localize ERO expression to prostate stroma cells (25–27).

Irrespective of the reference gene used, and although ERO mRNA level was detected to varying degrees, there is a reasonable trend showing that ERO is down-regulated in hormone-refractory tumors (Fig. 2B). Loss or down-regulation of ERO expression has been observed in CaP (5, 12). Moreover, an inverse correlation between ERO expression and histological grade or pathological stage has been frequently documented using ISH and IHC techniques (27, 28). ERO gene is extensively methylated in CaP cell lines and CaP tissues. The methylation levels correlate with tumor pathological grades and could inversely correlate with ERO gene expression (29). Low ERO expression was, furthermore, associated with poor prognosis for effective endocrine therapy (30). Our data, in agreement with these previous studies, strongly suggest that ERO expression is down-regulated in stroma cells of hormone-refractory tumors and consequently could have an indirect effect, through mesenchymal-epithelial interactions, on hormone dependence in CaP.

Although ERβ is expressed at high levels in secretory luminal cells of the rat and the monkey prostate glands (13–15), Bonkoff et al. (25) were unable to detect any reliable staining of ERβ in prostate tissue using the IHC assay. Our data suggest that the detection of ERβ mRNA in all of the prostate tissues analyzed reflects the higher sensitivity of the RT-PCR method compared with that of IHC.

ERβ appeared to be down-regulated in approximately one-half of
either localized or hormone-refractory tumors irrespective of the reference gene. Present knowledge of the distribution of this receptor in normal and neoplastic human prostate tissues is limited. The fact that ERα and ERβ may form functional heterodimers (31) suggests that ERβ expression, like ERα, could be stroma-cell dependent, whereas recent data from Lau et al. (32) indicate that, in normal epithelial prostatic cells, estrogen action is signaled via ERβ.

Our data indicate that decreased expression in ERβ could influence both stroma and epithelial cancer cell growth and, furthermore, support the idea that ERβ may normally protect against abnormal growth in keeping with the Chang and Prins (33) data showing that ERβ knockout mice display signs of prostatic hyperplasia with aging.

It is noteworthy that the androgen-refractory CaP cell line DU145, which expresses ERβ and not ERα, responded to antiestrogen by growth inhibition (32). This antiestrogen-induced growth-inhibitory response in DU145 cells was reversible by cotreatment with an ERβ antisense oligonucleotide. When our results are considered alongside those of Lau et al. (32), they support the central role played by ERβ and suggest that ERβ expression in prostate tumor can predict the response to antiestrogen treatment. Although ERα and ERβ expression are not correlated with treatment in hormone-refractory tumors, the tumor showing the highest expression of ERα and ERβ (T101) was obtained from a patient treated by LHRH agonists in keeping with the Chang and Prins (33) findings (34).

The role of PGR in human prostate has not yet been studied in detail. Previous data on PGR expression, especially those using IHC, are controversial (5–8). In this study, overall, PGR was obtained from a patient treated by LHRH agonists in keeping with the Chang and Prins (33) data showing that ERβ knockout mice display signs of prostatic hyperplasia with aging.

There is a clinical need for future compounds that produce a complete blockade of steroid hormone activity even in recurrent tumors. A comprehensive elucidation of steroid hormone receptor expression in hormone-refractory prostate tumors is an important first step in finding those compounds, the promising new tools that avoid drug-resistant phenotypes in clinical CaP therapy.

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