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

Alain Latil,1 Ivan Bieche, Dominique Vidaud, Rosette Lidereau, Philippe Berthon, Olivier Cussenot, and Michel Vidaud

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 endocrine treatment failure, it cannot be considered as 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.

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 as 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.

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 (pT2 N0 M0), whereas local extracapsular extension (without regional lymph node involvement) was observed in 5 (pT3 N0 M0); and (b) patients with metastatic disease, for which radical surgery is excluded, have been treated by endocrine therapy, either by classical androgen deprivation (orchiectomy or LHRH agonist) or by maximal androgen blockade (castration combined with 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 tumor 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.

**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:

\[ N_{\text{target}} = 2^{\frac{\Delta \text{Ct}_{\text{target}} - \Delta \text{Ct}_{\text{ref}}}{\text{Ct}_{\text{ref}}}} \]  

(A)

where ΔCt 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 gene. 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,

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotides Sequence</th>
<th>PCR product size (bp)</th>
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<tr>
<td>AR</td>
<td>Upper primer 5'-GCCGGCTTCGAAGAGTTCAC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lower primer 5'-GCCGGCTTCGAAGAGTTCAC-3'</td>
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<td>Lower primer 5'-GCCGGCTTCGAAGAGTTCAC-3'</td>
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<td></td>
<td>Lower primer 5'-GCCGGCTTCGAAGAGTTCAC-3'</td>
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<td></td>
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<td></td>
<td>Lower primer 5'-GCCGGCTTCGAAGAGTTCAC-3'</td>
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Table 1 Oligonucleotide primer sequences for real-time quantitative RT-PCR

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, ERa, ERβ, and PGR in human prostate tissues

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 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 products were purified and sequenced to confirm their 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.** RNA was reverse transcribed in a final volume of 20 μl containing 1× reverse transcriptase buffer [500 mM each dNTP, 3 mM MgCl_{2}, 75 mM KCl, and 50 mM Tris-HCl (pH 8.3)], 10 units of RNasin RNase inhibitor (Promega, Madison, WI), 10 mM DTT, 50 units of Superscript II reverse transcriptase, and 12 clinically prostate adenocarcinoma (T) samples ( ), and 11 hormone-refractory prostate tumors ( ). The expression levels are displayed as a ratio between the target gene and two reference genes (RPLP0, and KRT18 and/or VIM) to correct for variation in the amounts of RNA as well as of the specific marker for epithelial cells (KRT18) and stroma cells (VIM). The ratios for each type of analysis have been normalized in such a manner that the mean ratio of the 4 normal prostate samples equals a value of 1. 

**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). On the other hand, one hormone-refractory tumor (T101) showed a 7-fold increase in ERα expression (Fig. 2B).

**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).

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.** 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 (orchietomy 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 ERα and ERβ subtypes in both normal and neoplastic prostate tissues. The mean expression levels of ERα 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 ERα is truly down-regulated in clinically localized tumors and otherwise normally expressed in a component cell-independent fashion or that the ERα 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 ERα expression to prostate stroma cells (25–27).

Irrespective of the reference gene used, and although ERα mRNA level was detected to varying degrees, there is a reasonable trend showing that ERα is down-regulated in hormone-refractory tumors (Fig. 2B). Loss or down-regulation of ERα expression has been observed in CaP (5, 12). Moreover, an inverse correlation between ERα expression and histological grade or pathological stage has been frequently documented using ISH and IHC techniques (27, 28). ERα gene is extensively methylated in CaP cell lines and CaP tissues. The methylation levels correlate with tumor pathological grades and could inversely correlate with ERα gene expression (29). Low ERα expression was, furthermore, associated with poor prognosis for effective endocrine therapy (30). Our data, in agreement with these previous studies, strongly suggest that ERα 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 ER\(\alpha\) and ER\(\beta\) may form functional heterodimers (31) suggests that ER\(\beta\) expression, like ER\(\alpha\), 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 ER\(\beta\).

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

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

In conclusion, our data indicate that ER\(\alpha\) and ER\(\beta\) 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 ER\(\beta\) knockout mice display signs of prostatic hyperplasia with aging.

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 appeared to be down-regulated in clinically localized tumors when RPLP0 was used for normalization (Fig. 4A). However, this apparent down-regulation was absent when the stroma-specific cell gene, VIM, was used for normalization (Fig. 2B). This can be explained by the fact that PGR expression is stroma dependent and that normal prostate tissues are richer in this kind of cell than carcinoma samples, which, by definition, arise from epithelial cells. On the other hand, PGR displayed a wide range of expression in hormone-refractory tumors. Four tumors (T10, T14, T16, and T17) showed a decreased expression of PGR, whereas five (T11, T12, T60, T63, T90, T101, and T105) showed an increased expression. These differences in PGR expression were independent of the treatment; up- and down-regulation was observed both in patients treated by LHRH agonists and in those treated by antiandrogen. There is a significant positive association with ER\(\alpha\) expression (Fig. 7, A and B) in keeping with estrogen-regulation of PGR. There is also a positive association between PGR and ER\(\beta\) (Fig. 7, C and D). This result is consistent with the Lau et al. (35) data that suggest that the expression of testosterone plus estradiol-17 \(\beta\)-induced dysplasia in rat prostate is induced by PGR mRNA expression, likely mediated via ER\(\beta\) action. Our data are also consistent with the fact that PGR is an estrogen-regulated gene and, furthermore, indicate that ER\(\alpha\) and ER\(\beta\) could act in a synergistic fashion on human prostatic growth to activate PGR expression (i.e., T101, which showed the highest expression levels of both ER\(\alpha\) and ER\(\beta\), also showed the highest PGR expression level).

In conclusion, our data indicate that AR overexpression may be involved in the process leading to androgen independence of prostate tumors. However, the failure of various hormonal therapies to induce a stable remission of the disease would imply that androgens are not the sole actor in this unresolved dilemma. It is now clear that factors other than androgens are involved in the regulation of abnormal prostatic growth and that the microenvironment that includes both epithelial and stromal prostate cells represents a crucial site at which steroid hormones and peptide growth factors directly and reciprocally influence tumor development and growth. We observed a decreased expression level of ER\(\alpha\) and ER\(\beta\) in hormone-refractory prostate tumors. Taken together with the fundamental knowledge of ER-mediated transcription, these findings indicate that besides AR, abnormalities in ER\(\alpha\) and/or ER\(\beta\) expression may modulate the growth response of CaP to hormone withdrawal. ER\(\alpha\) and ER\(\beta\) expression status could, furthermore, be of primary importance for the treatment of advanced prostate tumors.

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.

ACKNOWLEDGMENTS

We thank Dr. David Grausz for fruitful collaboration in the reviewing of the manuscript.

REFERENCES


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