Prostate Carcinoma Expression of Estrogen Receptor-β as Detected by PPG5/10 Antibody has Positive Association With Primary Gleason Grade and Gleason Score

EMINA TORLAKOVIC, MD, WOLFGANG LILLEBY, MD, GORAN TORLAKOVIC, MD, SOPHIE D. FOSSÅ, MD, PHD, AND RAJNI CHIBBAR, MD, PHD

The objective of this study was to study the expression of estrogen receptor-β (ER-β) in prostatic adenocarcinoma and correlate it with Gleason grade and clinical outcome. Immunohistochemical evaluation was performed on prostate needle biopsies from 53 patients (T1-T3pN0M0). ER-β and ER-α transcripts were also studied by semiquantitative reverse transcriptase polymerase chain reaction in PC-3 and LNCaP prostate carcinoma cell lines. ERβ was expressed in 93% of adenocarcinomas and was positively associated with primary Gleason grade ($P = 0.028$ for percentage of positive cells and $P = 0.046$ using a semiquantitative scale) and Gleason score ($P = 0.010$ for percentage of positive cells and $P = 0.014$ using a semiquantitative scale). ER-β expression in the benign epithelium of prostates with adenocarinoma was detected in 92% of cases and in the stroma in 47% of cases. A trend for longer time to treatment failure was noted for cases with low ER-β expression after curatively intended radiotherapy ($P = 0.082$). PC-3, an aggressive prostate cancer cell line with invasive properties in nude mice, expressed higher levels of ER-β than LNCaP, a nonmetastasizing cell line, whereas no difference for ER-α transcripts could be observed. Our findings suggest that ER-β, as detected by PPG5/10 antibody, may have a role in the process of dedifferentiation of prostate adenocarcinomas, with higher levels present in less differentiated tumors. HUM PATHOL 33: 646-651. **Copyright 2002, Elsevier Science (USA). All rights reserved.**

Key words: **estrogen receptor-β, prostate, adenocarcinoma, Gleason grade, time to treatment failure.**

Abbreviations: **ER-β, estrogen receptor-β; SHBG, sex hormone–binding globulin; RT-PCR, reverse transcriptase polymerase chain reaction; G6PDH, glucose-6-phosphate dehydrogenase.**

The role of estrogen in pathogenesis of prostate cancer is not yet fully understood, and there is no direct evidence linking prostate cancer to estrogen. Prospective studies in men have shown no relation between prostate cancer and baseline serum concentration of total testosterone.[1,2] Prostate cancer arises later in life when circulating levels of free testosterone are declining because of increased plasma levels of sex hormone–binding globulin (SHBG). SHBG binds testosterone with greater affinity than estrogen.[3-6] Decline in the plasma free testosterone level is accompanied by an increase in the peripheral conversion of androgens to estrogens, altering the testosterone to estrogen ratio in favor of estrogen with increasing age.[6] In men, the relative risk of prostate cancer increases linearly with increasing serum levels of androstenidione and estradiol.[1] In addition, androstenidione can be converted to testosterone, estrone, and estradiol at a significantly higher rate in human prostatic tissue.[7] In Noble rats (the rat model for prostate cancer), estrogen has synergistic effect with androgen to initiate prostatic intraepithelial neoplasia (PIN)–like changes that are not inducible by androgen alone, suggesting that estrogens modify the effects of androgens on prostate tissue.[8] Although these studies do not provide unequivocal evidence of link between increased circulating androgen levels and prostate cancer risk, they do suggest a role for estradiol in the pathogenesis of PIN and prostate cancer.

The effects of estrogen are mediated via estrogen receptors (ERs). There are 2 types of ERs: ER-α, a well-characterized ER, and ER-β, a novel ER recently cloned from rat prostate epithelium with a different tissue distribution than ER-α.[9] The role of ER-β in the pathogenesis or prognosis of prostate cancer is unclear.[10,11] In prostate epithelium, ER-β is preferentially expressed in epithelial cells whereas ER-α is found in stromal and basal cells.[12] The presence of ER-α in epithelial cells is controversial.[13-15] Both types exhibit variable degrees of homology to one another, bind estrogen with similar affinity, and regulate some estrogen-dependent promoters similarly, but others in opposing fashion.[16,17]

In this study, we examined ER-β peptide as detected by PPG5/10 antibody in 53 prostate cancer biopsies and correlated it with Gleason grades, Gleason score, and clinical outcome. We also examined the relative expression of mRNA ER-β and ER-α by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) in LNCaP and PC-3, 2 well-characterized prostate cancer cell lines with different phenotypes and metastatic potentials.

From the Departments of Pathology and Oncology, Norwegian Radium Hospital, Oslo, Norway, and the Department of Pathology, Royal University Hospital, Saskatoon, Canada. Accepted for publication January 31, 2002.

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Address correspondence and reprint requests to Emina Emilia Torlakovic, MD, Department of Pathology, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway.

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MATERIALS AND METHODS

Patients

All samples were retrieved from the archives of the Department of Pathology, Norwegian Radium Hospital, Oslo, Norway. The study comprised 60 patients (median age, 66; range, 52 to 81) referred to the Norwegian Radium Hospital for definitive radiotherapy for localized T2/3pN0M0 prostate cancer between 1989 and 1995. The tumor category was mainly T3 (70%), and median pretreatment prostate-specific antigen level was 21 mg/mL (range, 2 to 86 mg/mL). From the 60 pretreatment needle biopsies, 53 specimens were judged by the pathologist to contain sufficient tissue for immunohistochemical analysis. None of the patients had received any hormonal manipulation before the needle biopsy. All needle biopsies and diagnostic pelvic lymphadenectomy were performed before the start of curatively intended radiotherapy. The method of conformal 4-field box technique has been described previously.[18] Briefly, all patients were given radiotherapy using a 4-field box technique (2 opposing anterior-posterior fields and 2 lateral portals) by a linear accelerator with 10- to 15-megavolt photons. The treatment volume was defined using a planning computed tomography scan as previously reported. The dose to the target volume prescribed was generally 66 Gy (range, 64 to 68 Gy; median, 66 Gy) according to the International Commission of Radiation Units (ICRU) reference point. Patients were followed for 19 to 142 months (median, 62 months). Progression was defined as the development of distant metastases, clinically detectable growth of the primary tumor, and/or rise of serum prostate-specific antigen level above 10 ng/mL. Time to progression was calculated from both the time of diagnostic needle biopsy and the start of curatively intended radiotherapy to the first sign or symptom of treatment failure or, in failure-free patients, to the last date of observation.

Gleason Grading

Prostate needle biopsies were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections were cut 4 to 6 μm thick, stained by routine hematoxylin and eosin, and reviewed by 2 pathologists (E.T. and G.T.) to confirm the diagnosis and assign Gleason grades according to previously published criteria.[19]

Immunohistochemistry

The paraffin blocks were cut 4 to 6 μm thick, dried overnight at 60°C, and deparaffinized in xylene. Then sections were rehydrated through graded alcohols into water. Heat-induced epitope retrieval was achieved by boiling sections in the EDTA buffer at pH 8.9 in an Electrolux microwave oven at 1000 W for 20 minutes (4 × 5 minutes). After boiling, sections were allowed to cool at room temperature for 20 minutes, rinsed thoroughly with water, and placed in Tris-buffered saline (TBS) for 5 minutes. Sections were incubated for 5 minutes with peroxidase block solution (EnVision; Dako, Glostrup, Denmark) to neutralize endogenous peroxidases. After washing with TBS, sections were incubated for 30 minutes at room temperature with mouse anti-human ER-β1, clone PPG5/10 (1:2 dilution; Serotec, Oxford, UK). Immunostaining was performed using the EnVision method (Dako) according to the manufacturer's instructions. Appropriate positive and negative controls were used. To determine the specificity of PPG5/10 antibody, the anti–ER-β antibody was preincubated with 0.2 μg/mL, 2 μg/mL, and 20 μg/mL peptide (recombinant human long-form ER-β1 and recombinant human ER-α; PanVera, Madison, WI) for 2 hours at room temperature and overnight at 4°C.

Scoring for the degree of intensity of the immunostained nuclei was done semiquantitatively (0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining). The percent positive nuclei of neoplastic cells were counted using a manual cell counter (Assistant Counter AC-8, Karl Hetch GmbH and Co., Sondheim, Germany) in 500 tumor cells. The percentage values were not categorized for the linear-by-linear association analysis. For the failure-free survival analysis, the results were categorized into 2 groups (<50% positive cells and ≥ 50% positive cells).

Cell Culture

PC-3 and LNCaP prostate adenocarcinoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). LNCaP and PC-3 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all from Life Technologies, Grand Island, NY).

Reverse Transcriptase Polymerase Chain Reaction

The sense and antisense primer pairs were synthesized at the DNA Laboratory, University of Alberta, Edmonton, Alberta, Canada. The primer sequences were as described previously.[20,21] To perform radioactive RT-PCR, the oligonucleotides were end-labeled using 32Pγ-ATP. For the first-strand complementary DNA synthesis, 1 μg of total RNA was reverse-transcribed with Superscript I reverse transcriptase (Gibco BRL, Grand Island, NY), using hexamers in a reaction volume of 80 μL. Five μL of this cDNA mixture was initially amplified for glucose-6-phosphate dehydrogenase (G6PDH) for 28 cycles, analyzed on 6% polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat AR (XAR) film (Amersham Biosciences, Quebec, Canada) for 12 hours. After confirming equal amounts of RNA in each sample, PCR amplification for ER-α and ER-β were performed on a thermal cycler (PerkinElmer, Boston, MA) using 25 μL of total reaction volume containing 5 μL of reverse-transcribed cDNA mixtures, 1.25 mM deoxyribonucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.2 U Taq polymerase (platinum Taq), and 5 pmole of each primer. The mixtures were subjected to 30 cycles of denaturation (1 minute at 94°C), annealing (30 seconds at 61°C), and extension (45 seconds at 72°C), with a final incubation for 10 minutes at 72°C. Six μL of the reaction product was electrophoresed on 6% polyacrylamide gel. The gels were dried and exposed to XAR film with an intensifying screen for 12 hours. The expression of the steroid receptors was compared to G6PDH, a housekeeping gene, and to each other.

Statistical Analysis

Association between the intensity of staining (semiquantitative scale) and the percentage of positive tumor cells was assessed using a linear-by-linear association test, as were associations between the intensity of imunohistochemical staining and Gleason grade and score. Overall survival curves were drawn by the Kaplan–Meier method.[22] The difference in curves was tested by the log-rank test.[23] P values <0.05 were considered significant.

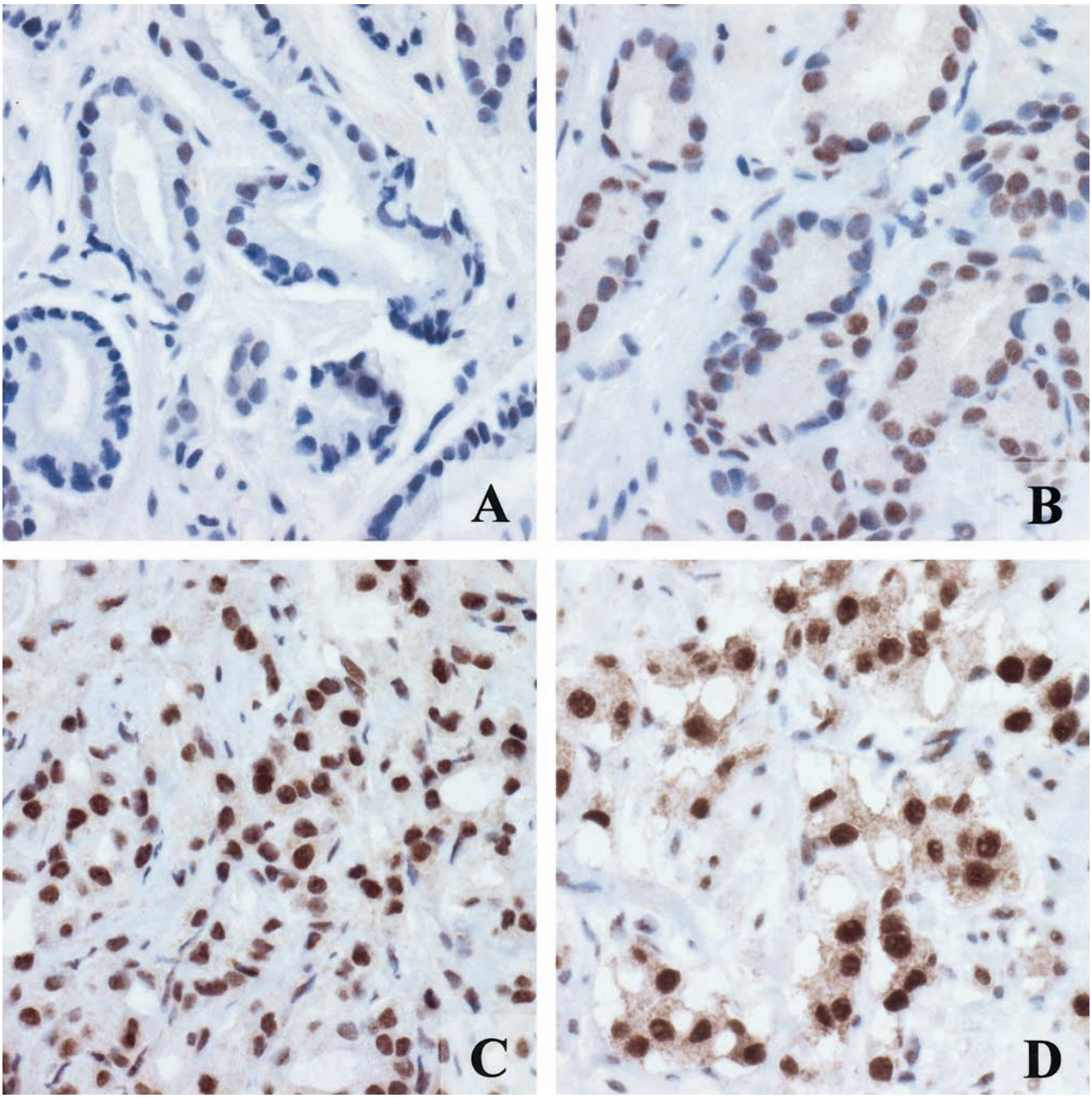

FIGURE 1. Immunohistochemical localization of ER-β using PPG5/10 antibody. (Detection by the EnVision method; original magnification $\times$400.) (A and B) Weak expression of ER-β in Gleason grade 3/3 prostate adenocarcinoma. In (A), groups of weakly positive nuclei are present. (C) and (D) Strong expression in Gleason grade 4/4 prostate adenocarcinoma. Several stromal cells were also positive in some cases, but in much lesser degree. The intensity of staining was scored as follows: 1+ in (A), 2+ in (B), and 3+ in (C) and (D).

RESULTS

Prostate Adenocarcinoma

Immunoreactivity for anti–ER-β was found in the nuclei of epithelial cells of prostate carcinoma (Fig 1). Cytoplasmic reactivity was occasionally observed in malignant epithelial cells; however, only nuclear staining was graded and recorded. Reactivity for the PPG5/10 antibody was successfully blocked by preincubation of the antibody with ER-β peptide using 20 μg/mL and 2 μg/mL concentrations, but not by preincubation with ER-α peptide at any concentration. Blocking was not observed with the lowest concentration of ER-β peptide (0.2 mg/mL).

ER-β was expressed in 49 of 53 (93%) of prostate carcinomas. The percentage of positive tumor cells and the intensity of staining showed strong positive association ($P < 0.0001$). There was a statistically significant

TABLE 1. Association Between Primary Gleason Grade and Staining Intensity Using Anti–ER-β Antibody (Clone PPG5/10)

Primary Gleason grade	ER-β immunoreactivity in tumor cells (%)				
	Negative	1+	2+	3+	Total
2		1 (50)	1 (50)		2
3	3 (14)	12 (53)	6 (28)	1 (5)	22
4	1 (4)	11 (39)	15 (53)	1 (4)	28
5				1 (100)	1
Total (%)	4 (8)	24 (45)	22 (42)	3 (5)	53 (100)

NOTE: $P = 0.046$, linear-by linear association.

TABLE 2. Association Between Gleason Score and Staining Intensity Using Anti–ER-β Antibody (Clone PPG5/10)

Gleason score	ER-β immunoreactivity in tumor cells (%)				
	Negative	1+	2+	3+	Total
4		2 (100)			2
5			1 (100)		1
6	2 (20)	7 (70)	1 (10)		10
7	1 (5)	8 (42)	9 (47)	1 (5)	19
8	1 (5)	7 (35)	11 (55)	1 (13)	20
9				1 (100)	1
Total (%)	4 (7)	24 (45)	22 (42)	3 (6)	53 (100)

NOTE: $P = 0.01$, Linear-by-Linear Association

positive association between the percentage of positive tumor cells and primary Gleason grade ($P = 0.028$) and Gleason score ($P = 0.010$). Similarly, the intensity of staining of neoplastic epithelial cells (measured on a semiquantitative scale) was positively associated with primary Gleason grade (Table 1) ($P = 0.046$, linear-by-linear association) and Gleason score (Table 2) ($P = 0.014$, linear-by-linear association).

A trend for longer failure-free survival time was found for cases with low expression of ER-β (< 50% cells positive), but statistical significance was not reached ($P = 0.082$) (Fig 2).

Prostatic Stroma and Benign Epithelium

The results are summarized in Table 3. Generally, no definite difference in staining intensity was seen between stromal cells surrounding prostate carcinoma cells and stromal cells surrounding benign glands. ER-β expression in the stroma was detected in 25 of 53 (47%) biopsies and was graded as weak in 24 (45%) positive biopsies and as moderate in 1 (2%) biopsy. No significant association between the level of ER-β expression in the stroma and Gleason grades was detected on the linear-by-linear association test. Benign epithelium was present in 51 of 53 biopsies. There also was no significant association between the level of ER-β expression in the benign epithelium and Gleason grades using the linear-by-linear association test.

Cell Lines

The results are shown in Figure 3. High levels of ER-β expression were detected in PC-3 cells, but almost none in LNCaP cells. There was no appreciable difference in ER-α transcripts between the 2 cell lines.

DISCUSSION

Contradictory reports on expression of ER-β in benign and malignant prostate tissue have recently been published.[10,13,24] Some studies have shown decreased or no expression of ER-β in prostate carcinoma,[24,25] others have shown definite expression and

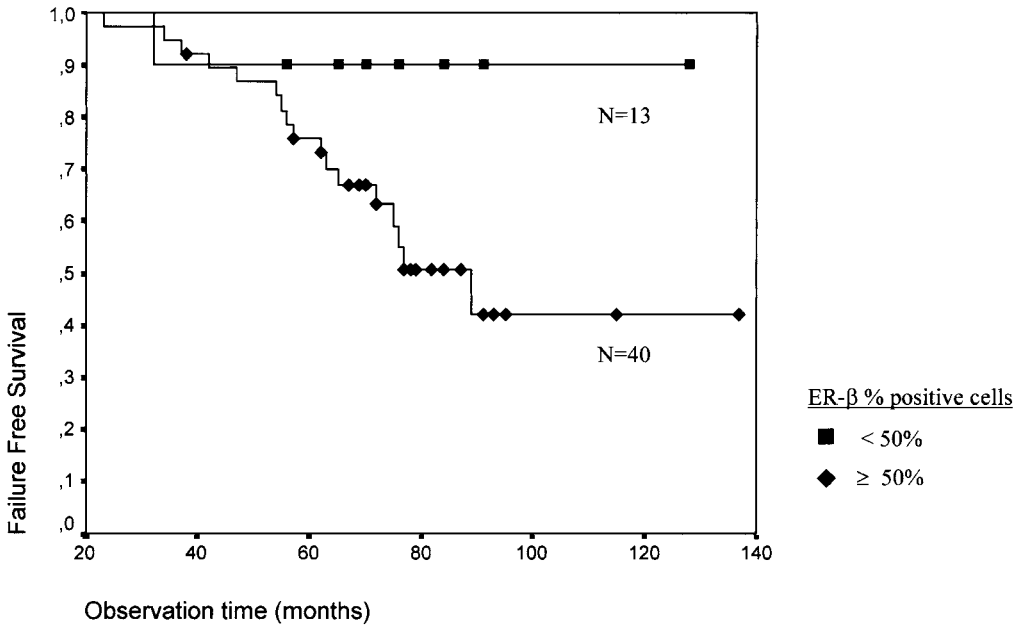

FIGURE 2. Failure-free survival of prostate carcinoma patients with low (<50% positive tumor cells) and high (≥50% positive tumor cells) expression of ER-β.

TABLE 3. Expression of ER-β in Prostate Stroma and Benign Epithelium

	Benign prostate	
ER-β intensity of staining	Stroma n = 53 (%)	Epithelium n = 51 (%)
0	28 (53)	4 (8)
1+	24 (45)	27 (53)
2+	1 (2)	18 (35)
3+		2 (4)

even increased expression,[13] and still others have shown both decreased and increased expression, depending on the stage/grade of prostate cancer.[10] ER-β RNA transcripts were found to be decreased in both localized and hormone refractory prostate cancers relative to normal prostate tissue when measured by quantitative RT-PCR. The latter observation suggested that the loss of ER-β could correlate with disease progression.[26] However, recent studies by Leav et al[10] and Royuela et al[13] showed ER-β expression in a large number of primary prostate carcinomas as well as in metastatic tumors. In our study, PPG5/10 antibody frequently detected ER-β in prostate carcinoma and showed a linear-by-linear association between the level of ER-β expression in malignant prostate epithelial cells and Gleason score and primary Gleason grade.

The presence of higher levels of ER-β transcripts in the highly aggressive cell line PC-3, as compared to the LNCaP cells, which is analogous to well-differentiated prostate carcinoma, parallels our results obtained in needle biopsies. Our findings in cell lines are similar to findings of Lau et al.[27]

There is no obvious explanation as to why the results reported in the literature on the levels of either ER-β mRNA or ER-β peptide are controversial. Immunohistochemical results depend on several factors, including at least tissue fixation and processing, antigen retrieval technique, antibodies and methods used for immunostaining and visualization, as well as the presence of known and unknown polypeptide variants.[28] The last possibility is of great significance, because some of the antibodies could be detecting nonfunctional variants in addition to the functional polypeptides. Because of this possibility, we have limited our conclusions solely to expression of ER-β as detected by PPG5/10 antibody. In addition to nuclear localization, cytoplasmic reactivity was also observed in occasional tumors, as well as in occasional benign epithelial cells. If this phenomenon reflects true ER-β that was not transported into the nuclei, then it could cause major confusion about both protein and mRNA expression, because only nuclear localization of the protein enables transcriptional activity of the protein. Only immunohistochemical analysis both identifies the protein and specifies its exact localization in the cells.

Ehara et a[29] suggested that half-lives of ER polypeptides vary depending on the physiologic states of cells, and that this could be a reason behind the conflicting reports in the literature. Even though the amounts of protein and mRNA transcripts do not always correlate perfectly, these authors suggested that protein expression and subcellular localization might be more representative of the protein activity/function than the amount of mRNA transcripts.[30] This is why immunohistochemical studies are so important. Bonkhoff et al[14] could not detect ER-β polypeptide in benign or malignant prostate. Their results are in contrast to most of the other published reports using both immunohistochemistry and RT-PCR methods, which show ER-β polypeptides and transcripts in both benign[10,13,24,31,32] and malignant prostate tissue.[10,13]

Primary diagnostic needle biopsy for immunohistochemical studies of prostate carcinoma has both advantages and disadvantages. The fixation of the tissue is rapid and very similar for each specimen, because the tissue is of uniform thickness, in contrast to most prostatectomy specimens, which are cut after the whole organ has been fixed. However, the small size of the specimen has its limitations, and focal events could be missed. An additional advantage of primary diagnostic needle biopsy is that the patients did not receive any treatment before the biopsy.

In summary, we observed that ER-β is expressed in prostate carcinoma, but, like in benign prostate tissue, it has a predilection for epithelial cells. We conclude that in prostate carcinoma ER-β as detected by

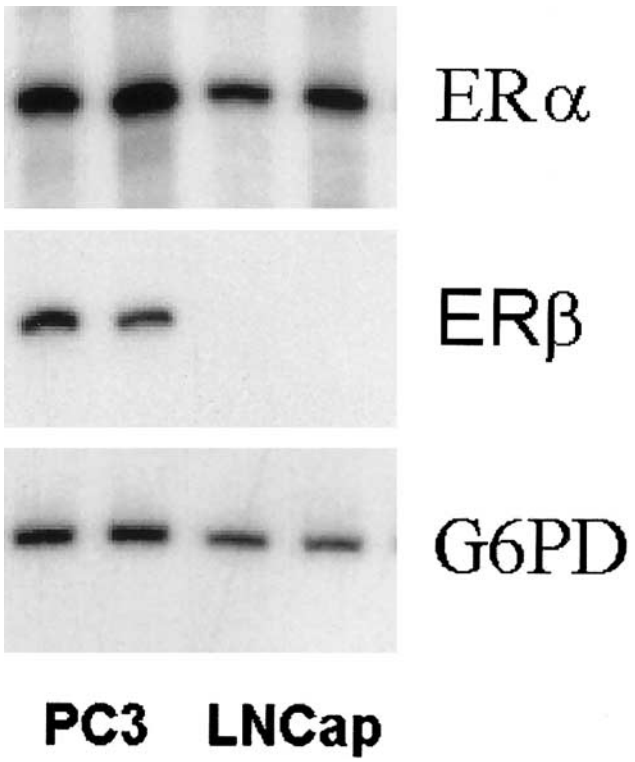

FIGURE 3. Semiquantitative RT-PCR. Comparison of ER-α and ER-β in prostate carcinoma cell lines PC-3 (invasive cell line), and LNCaP (noninvasive cell line). Expression of ER-β transcripts is higher in PC-3 than in LNCaP; no differences in ER-α transcripts were identified. Amplified PCR products were analyzed on 2% agarose gel.

PPG5/10 antibody is frequently present, and its expression linearly correlates with primary Gleason grade and Gleason score. We suggest that ER-β could have a role in prostate carcinoma dedifferentiation. Even though in this limited study the expression of ER-β was not found to significantly correlate with outcome, a trend for longer time to treatment failure was noted for cases with low ER-β expression after curatively intended radiotherapy.

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