Expression and Nuclear Localization of ErbB3 in Prostate Cancer

Ismaël Hervé Koumakpayi,1 Jean-Simon Diallo,1 Cécile Le Page,1 Laurent Lessard,1 Martin Gleave,5 Louis R. Bégin,2 Anne-Marie Mes-Masson,1,3 and Fred Saad1,4

Abstract

Purpose: The ErbB1 and ErbB2 receptors have been implicated in prostate cancer progression, but less is known about the role and biology of other ErbB receptor family members in prostate cancer. The aim of this study was to analyze the expression and localization of ErbB3 in prostate tissues and prostate cancer cell lines.

Experimental Design: Immunohistochemistry of ErbB3 was done on prostate cancer tissue sections from 143 patients and on a tissue microarray containing 390 cores of radical prostatectomy-derived specimens representing normal, prostatic intraepithelial neoplasia, and malignant tissues from 81 patients. ErbB3 subcellular localization was studied by Western blot analysis in LNCaP, 22Rv1, PC-3, and DU145 prostate cancer cell lines.

Results: Immunohistochemistry analysis of prostate cancer tissues revealed that >90% of prostate cancer tissues displayed cytoplasmic ErbB3 staining. Minimal ErbB3 nuclear staining was observed in normal prostate tissues and benign prostatic hyperplasia tissues; in contrast, ErbB3 was frequently localized in the nucleus of cancerous tissues. This nuclear localization was more frequent (P < 0.001) in hormone-refractory tissues (17 of 17, 100%) compared with hormone-sensitive samples (37 of 92, 40.2%). Additionally, in the tissue microarray, increased nuclear ErbB3 was associated with increasing Gleason grade. Interestingly, Western blot analysis of cytoplasmic and nuclear subcellular fractions showed that ErbB3 nuclear localization was more prevalent in hormone-sensitive prostate cancer cell lines (LNCaP and 22Rv1) compared with hormone-insensitive cell lines (PC-3 and DU145).

Conclusions: ErbB3 nuclear localization discriminates normal from malignant prostate tissues and between tumors from hormone-sensitive versus hormone-refractory prostate cancer. ErbB3 nuclear staining seems to be associated with risk of disease progression. The high frequency of ErbB3 nuclear localization in hormone-refractory tissues indicates that ErbB3 warrants further study to understand its association with prostate cancer disease progression.

Prostate cancer is the second leading cause of cancer death among North American men. In 2003, prostate cancer was the most frequently diagnosed cancer in men, accounting for 30% of newly diagnosed cases and 10% of deaths (1). Improving the treatment of prostate cancer will require early diagnosis and appropriate therapeutic decisions based on a better understanding of the biology of prostate cancer and the underlying molecular events that predict disease progression.

The ErbB receptor family consists of four receptors: ErbB1/epidermal growth factor receptor (EGFR), ErbB2/Her2, ErbB3, and ErbB4 (2, 3). ErbB signaling produces a diversity of responses generated by three levels of molecular interactions. The first is ligand-receptor binding, the second is homodimerization and heterodimerization, and the third is associated with distinct sets of cytoplasmic signaling proteins largely dependent on the nature of the active dimer formed (4). Previous studies have shown that ErbB receptor activation promotes multiple tumorigenic processes, such as proliferation, angiogenesis, metastasis, and resistance to apoptosis (5, 6).

Because there is growing evidence for a role of growth factors in the development and progression of many human cancers, ErbB receptors have been studied for their use as potential prognostic markers and as therapeutic targets (7). EGFR overexpression is usually oncogenic in the presence of ligand. In several cancers, including prostate cancer, the association between elevated EGFR levels and poor clinical outcome is

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particular clear (8, 9). The ErbB2 receptor is also oncogenic. Compared with other ErbB receptors, ErbB2 is a potent ligand independent activator of the mitogen-activated protein kinase pathway (4). In prostate cancer, ErbB2 expression correlates even more closely with clinical outcome than does EGFR expression (10). ErbB4 receptor tyrosine kinase activity is associated with differentiation, growth arrest, and tumor suppression (11). ErbB4 is generally not expressed in prostate cancer cell lines except in the hormone-sensitive 22RV1 cell line (12–14), and only ~20% of prostate cancer tissues express ErbB4 (15, 16). In contrast, only a small number of studies have addressed the expression and the role of ErbB3 in prostate cancer cell lines and more particularly in prostate cancer tissues.

The ErbB3 gene is located on chromosome 12q11-13. ErbB3 mRNA gives rise to a 185-kDa transmembrane glycoprotein with the same basic features as the other members of the ErbB family and is composed of three regions: an NH2-terminal extracellular ligand-binding region, a transmembrane domain, and an intracellular region containing the COOH-terminal tyrosine kinase domain (17, 18). However, due to a mutation in the tyrosine kinase domain, ErbB3 lacks intrinsic kinase activity but can be transphosphorylated and can transduce a signal by heterodimerizing with another tyrosine kinase receptor. ErbB3 can potentially heterodimerize with each of the three other ErbB members as well as with the interleukin-6 receptor subunit gp130 (2, 19); however, the ErbB2/ErbB3 heterodimer potentiates the strongest downstream signaling response.

ErbB3 mRNA expression has been detected in various tissues, such as placenta, skin, stomach, endocrine and nervous system, and in the kidney and intestines (18, 20). ErbB3 protein is expressed in normal human tissues and in several cancers (20–24). Many studies have observed either the overexpression or gene amplification of ErbB3 in different carcinomas and cancer cell lines (24–27) and more particularly in breast cancer cell lines and breast cancer tissues (18, 28). Its high expression is associated with short patient survival in breast cancer (29), oral squamous cell carcinomas (30), and lung cancer (31). In situ ductal carcinoma of the breast, normal levels of membrane and cytoplasmic expression of ErbB3 coincide with overexpression of ErbB2, whereas overexpression of ErbB3 correlates with decreased expression of ErbB2 (32). Notably, ErbB3 has been observed to preferentially heterodimerize with ErbB2 in several cancers, leading to a strong oncogenic signal thought to promote tumor cell proliferation (24, 26, 28). Even if in prostate cancer cell lines ErbB3 is often coexpressed with ErbB1 and ErbB2 (12), surprisingly, these receptors were not found to heterodimerize in hormone-sensitive (LNCaP) and hormone-insensitive (PC-3 and DU145) prostate cancer cell lines (14).

Whereas the contribution and the role of ErbB1 and ErbB2 in prostate cancer have been extensively studied, the contribution of ErbB3 and its role in prostate cancer progression remain unclear. Indeed, studies done on a small number of cases have previously looked at ErbB3 in prostate cancer and have come to conflicting conclusions (15, 16, 22, 33). In parallel, recent studies showed that membranous ErbB1, ErbB2, ErbB3, and ErbB4 proteins can translocate to the nucleus in a variety of cancer cells (34–38), and that this event may play an important role in cancer progression. As subcellular localization was not taken into account in previous studies, it is possible that discrepancies related to ErbB3 expression analysis in prostate cancer tissues could in part be due to this confounding factor.

The aim of this study was initially to analyze the expression and localization of ErbB3 in normal and cancerous prostate tissues and in cell lines. In tissues, we found that ErbB3 was frequently localized in the nucleus of prostate cancer cells but rarely in normal cells. Additionally, the frequency of ErbB3 nuclear localization increased with pathologic stage, being most frequent in hormone-refractory prostate cancer tissues.

Materials and Methods

Tissue samples and tissue array. One hundred forty-three formalin-fixed, paraffin-embedded, archival tissues from patients from the Centre hospitalier de l’Université de Montréal Notre-Dame hospital were included in this study (Table 1). Of those 143 samples, 92 were from hormone-sensitive patients that had undergone radical prostatectomy, and none of these radical prostatectomy patients received androgen deprivation therapy before surgery. Forty-four specimens were obtained by transurethral resection of the prostate from 17 hormone-refractory patients and 27 benign prostatic hyperplasia (BPH) patients, and seven samples were normal prostate specimens obtained from autopsies.

The tissue array was made at the Prostate Centre of Vancouver General Hospital and consists of 390 cores of radical prostatectomy-derived, formalin-fixed specimens of normal, high-grade prostatic intraepithelial neoplasia (PIN), and cancerous prostate tissues from 81 patients representing different Gleason grades of the disease. The tissue array was divided into five groups of cores. Group 1 contained 43 normal tissue cores from areas adjacent to cancer. Group 2 contained 25 PIN cores from patients with cancer. Group 3 contained 200 low Gleason grade cores (Gleason 2 and 3). Group 4 consisted of 115 high Gleason grade cores (Gleason 4 and 5). Group 5 was composed of seven cores from hormone-refractory patients (Table 2).

Immunohistochemistry. Samples were immunostained with a rabbit polyclonal anti-ErbB3 antibody (C-17, Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution = 2 μg/ml) recognizing the COOH-terminal portion of ErbB3, with a mouse monoclonal anti-ErbB3 antibody (RTJ-2, Santa Cruz Biotechnology; 1:100 dilution = 2 μg/ml) recognizing the cytoplasmic domain, or with a rabbit polyclonal anti-ErbB3 antibody (Ab-9, Neomarkers, Fremont, CA; 1:50 dilution = 4 μg/ml) recognizing the NH2-terminal domain. Primary antibody detection was done using the LSAB 2 peroxidase system from DAKO Inc. (Carpinteria, CA). Staining was done as described previously (39). Briefly, tissue samples were deparaffinized, rehydrated, and treated with 0.3% H2O2 in methanol to eliminate endogenous peroxidase activity. An antigen retrieval step was done using 10 mmol/L citrate buffer (pH 6) applied for 15 minutes at 95°C. All following steps were done at room temperature. The sections were then blocked with a protein-blocking serum-free reagent (DAKO) and incubated with anti-ErbB3 antibody for 60 minutes followed by a 20-minute treatment with the secondary biotinylated antibody (DAKO) and then incubated for 20 minutes with streptavidin-peroxidase (DAKO). Reaction products were developed with diaminobenzidine (DAKO) containing 0.3% H2O2 as a substrate for peroxidase. Nuclei were counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO).

Scoring procedure and statistics. Brown-colored ErbB3 cytoplasmic and nuclear staining in tissue samples was visualized by light microscopy and evaluated independently analyzed by two blinded observers. All tissue sections were given continuous values representing the percentage of cells exhibiting nuclear ErbB3 staining. Categories were determined according to the median of cancerous tissues samples scored, and the samples in which the percentage of cancer cells exhibiting nuclear ErbB3 staining exceeded the median were counted as positive (40). Staining...
Table 1. Clinical and pathologic details of patients according to ErbB3 nuclear staining status

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>ErbB3 ≥ median*, n (%)</th>
<th>ErbB3 &lt; median*, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>0 (0)</td>
<td>7 (100)</td>
<td>1.3 × 10^{-10}</td>
</tr>
<tr>
<td>BPH</td>
<td>27</td>
<td>0 (0)</td>
<td>27 (100)</td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>17</td>
<td>17 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>HS</td>
<td>92</td>
<td>37 (40.2)</td>
<td>55 (59.8)</td>
<td>0.672</td>
</tr>
<tr>
<td>Age, mean (SE)</td>
<td>62.02 (0.89)</td>
<td>62.90 (0.63)</td>
<td>0.581</td>
<td></td>
</tr>
<tr>
<td>Preoperative PSA, mean (SE)</td>
<td>11.80 (1.41)</td>
<td>9.96 (0.85)</td>
<td>0.581</td>
<td></td>
</tr>
</tbody>
</table>

Gleason sum

| <7         | 54  | 25                     | 29                     | 0.569   |
| 7          | 27  | 10                     | 17                     |         |
| >7         | 11  | 6                      | 5                      |         |

Resection margins

| Negative   | 37  | 19                     | 18                     | 0.195   |
| Positive   | 55  | 22                     | 33                     |         |

Metastases

| Negative   | 84  | 36                     | 48                     | 0.283   |
| Positive   | 8   | 5                      | 3                      |         |

Abbreviations: PSA, prostate-specific antigen; HR, hormone refractory; HS, hormone sensitive; n, number of patients.
*Pearson χ² tests, median = 24%.

Intensities were separated into five classes: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining; and 4, very strong staining. All statistical tests were done using Statistical Package for the Social Sciences version 11 (SPSS, Inc., Chicago, IL).

**Cell culture and reagent.** Prostate cancer cell lines LNCaP, PC-3, DU145, and 22Rv1 cells were incubated at 37°C, 5% CO₂ in RPMI 1640 (Wisent, Inc., St. Jean Baptiste de Rouville, Quebec, Canada) supplemented with 10% of fetal bovine serum (Hyclone, Logan, UT). The RWPE-1 cell line, derived from human normal prostate epithelial cells and immortalized by the human papilloma virus 18, was incubated at 37°C, 5% CO₂ in keratinocyte media (Invitrogen, Carlsbad, CA). The human breast cancer cell line MCF-7 was used as a positive control for ErbB3 phosphorylation.

**Western blot analysis.** Protein extracts were boiled in loading buffer, separated by 8% SDS-PAGE, and transferred to nitrocellulose membrane (Bio-Rad Lab) under refrigerated conditions at 200 mA for 2 hours. Membranes were blocked for 30 minutes in 5% milk/PBS/0.1% Tween 20, incubated for 2 hours at room temperature with specific antibodies (0.5-1 μg/mL), washed twice with PBS/0.05% Tween 20 and incubated for another 30 minutes at room temperature with peroxidase-conjugated antibodies (Santa Cruz Biotechnology). Immunodetection was done as described in the enhanced chemiluminescence detection reagent kit (Amersham Biosciences, Buckinghamshire, United Kingdom). Polyclonal anti-ErbB3 (C-17, Santa Cruz Biotechnology).

**Whole cell, cytoplasmic, and nuclear extracts.** For whole-cell extracts, cells were lysed as described previously (14) with cold lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L NaF, 0.5% NP40, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 2 μg/mL of aprotinin, leupeptin, and pepstatin]. Lysates were centrifuged for 15 minutes (13,000 rpm at 4°C), and the supernatants containing whole-cell extracts were carefully collected and stored. Protein concentration was determined using the Bradford method (Bio-Rad Lab, Hercules, CA).

**Cytoplasmic and nuclear fractions.** Cytoplasmic and nuclear fractions were obtained as follows: cells were washed twice with cold PBS, centrifuged for 10 minutes (1,500 rpm at 4°C), and resuspended in 300 μL of cell lysis buffer [10 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 10 mmol/L EDTA, 5 mmol/L MgCl₂] supplemented with protease inhibitors. Cells were kept on ice for 30 minutes to allow swelling, and 0.1% NP40 was subsequently added for 10 minutes, after which 10% glycerol (v/v) was added, and lysed cells were centrifuged for 5 minutes (1,000 rpm at 4°C). The supernatants containing cytoplasmic extracts were carefully removed, aliquoted, and stored. The nuclear pellets were resuspended for 1 hour on ice in 40 μL of nuclear lysis buffer [10 mmol/L HEPES (pH 7.9), 50 mmol/L NaCl, 0.1 mmol/L EDTA, 0.5 mmol/L DTT, 5% glycerol] supplemented with protease inhibitors. Particulate matter was eliminated by centrifugation for 10 minutes (13,000 rpm at 4°C), and the supernatants containing nuclear extracts were carefully collected and stored. Protein concentration was determined using the Bradford method (Bio-Rad Lab).

**Table 2. ErbB3 total staining in prostate tissue array**

<table>
<thead>
<tr>
<th>Group</th>
<th>Negative (%)</th>
<th>Positive (%)</th>
<th>P (Kruskal-Wallis test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3/43 (7)</td>
<td>40/43 (93)</td>
<td></td>
</tr>
<tr>
<td>PIN</td>
<td>1/25 (4)</td>
<td>24/25 (96)</td>
<td></td>
</tr>
<tr>
<td>LG</td>
<td>18/200 (9)</td>
<td>182/200 (91)</td>
<td>0.375</td>
</tr>
<tr>
<td>HG</td>
<td>6/115 (5)</td>
<td>109/115 (85)</td>
<td>0.002</td>
</tr>
<tr>
<td>HR</td>
<td>1/7 (14.3)</td>
<td>6/7 (85.7)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: LG, low Gleason grade; HG, high Gleason grade; HR, hormone refractory.
monoclonal anti-ErbB3 (RT2, Santa Cruz Biotechnology), polyclonal anti-ErbB3 (RB-9211, Neomarkers), monoclonal anti-α-tubulin (TU-02, Santa Cruz Biotechnology), polyclonal anti-phospho-ErbB3 (Tyr\textsuperscript{1298}, # 4791, Cell Signaling, Beverly, MA), and monoclonal anti-β-actin (ab2272, Abcam, Cambridge, MA) primary antibodies were used. All experiments were repeated at least twice to confirm similar results. The densitometry analysis used for ErbB3/actin ratio was done using Image-Pro Plus (Media Cybernetics, Silver Spring, MD) software.

**Results**

**ErbB3 expression in prostate tissues.** Immunohistochemistry was used to analyze the protein expression of ErbB3 in prostate specimens. Staining was done on a set of 143 tissue sample sections (Table 1) and on a tissue array containing 390 cores (Table 2) from normal, BPH, hyperplastic, and cancerous prostate tissues representing all prostate cancer Gleason grades. Almost all of the 143 tissue sample sections displayed cytoplasmic ErbB3 staining. Similarly, in the tissue array, ErbB3 cytoplasmic staining was observed in about 90% of cores (Table 2; Fig. 1). The proportion of stained cells was not significantly different among the histopathologic grades ($P = 0.375$, Kruskal-Wallis test; see Table 2). In addition, the average staining intensity did not vary considerably between the subgroups (data not shown). Surprisingly, we noticed that ErbB3 localized to the nucleus of prostate cancer cells (Table 3; Fig. 1C and D) in both the tissue sections and the tissue array. To confirm the validity of the observed nuclear staining, we used two other ErbB3-specific antibodies (RT2 and Ab-9). All three antibodies gave similar patterns (data not shown), thus validating our observation of nuclear ErbB3 localization in prostate cancer tissues.

**ErbB3 expression and localization in prostate cancer cell lines.** Others have previously observed the nuclear localization of full-length ErbB3 in breast cancer cell lines by immunofluorescence (36). To confirm that ErbB3, a membrane protein, could be localized in the nucleus of prostate cancer cells, the subcellular localization of ErbB3 was further tested by Western blotting on cytoplasmic and nuclear enriched fractions of LNCaP, 22Rv1, PC3, DU145, and RWPE-1 cell lines (Fig. 2A and Fig. 3B and C). The fractions were probed for presence of cytoplasmic marker α-tubulin to evaluate the purity of nuclear extracts (Fig. 2A and Fig. 3C) and revealed no detectable cytoplasmic proteins in nuclear fractions. Interestingly, as shown in Fig. 2A and Fig. 3C, although all cell lines exhibited nuclear ErbB3, the androgen-sensitive cell lines LNCaP and 22Rv1 displayed the most ErbB3 in nuclear extracts compared with androgen-insensitive PC-3 and DU145 cells. Overall, whole-cell extracts also showed higher expression of ErbB3 in LNCaP and 22Rv1 compared with PC-3 and DU145 prostate cancer cells (Fig. 2B and Fig. 3A). Densitometric analysis of the ratio of ErbB3/actin confirmed these observations. In contrast, the normal prostate cell line RWPE-1 exhibited low levels of expression and nuclear localization of ErbB3 (Fig. 3A-C).

**Neuregulin1-β1 binds directly to ErbB3 and ErbB4 tyrosine kinase receptors and results in tyrosine residue phosphorylation** (3, 4). In addition, neuregulin1-β1 treatment has previously been reported to affect the subcellular localization of ErbB3 in human mammary cell lines MTSV1-7 and MCF-7 by inducing its translocation to the nucleus (36). In whole-cell extracts, LNCaP and 22Rv1 cells exhibited strong ErbB3 phosphorylation compared with PC-3, DU145, and RWPE-1 cells upon neuregulin1-β1 stimulation (Fig. 3A). To address the phosphorylation status of ErbB3 in cytoplasmic and nuclear fractions of prostate cancer cells, we stimulated cells with neuregulin1-β1

### Table 3. ErbB3 nuclear staining in prostate tissue sections

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>BPH</th>
<th>HS</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean of % (SD)</td>
<td>3.5 (4.1)</td>
<td>0.87 (0.73)</td>
<td>24.4 (15.1)</td>
<td>63.6 (16.9)</td>
</tr>
<tr>
<td>Using median (24%) as cutoff</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative (%)</td>
<td>7/7 (100)</td>
<td>27/27 (100)</td>
<td>55/92 (59.8)</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>Positive (%)</td>
<td>0/7 (0)</td>
<td>0/27 (0)</td>
<td>37/92 (40.2)</td>
<td>17/17 (100)</td>
</tr>
</tbody>
</table>

Fisher’s exact test, $P$

<table>
<thead>
<tr>
<th>Group</th>
<th>BPH</th>
<th>HS</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPH</td>
<td>ND</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HS</td>
<td>0.043</td>
<td>1.0 × 10^{-6}</td>
<td>—</td>
</tr>
<tr>
<td>HR</td>
<td>2.89 × 10^{-6}</td>
<td>1.46 × 10^{-12}</td>
<td>1.44 × 10^{-6}</td>
</tr>
</tbody>
</table>

Abbreviations: HS, hormone sensitive; HR, hormone refractory; ND, not determined.
hormone-sensitive tissues, 54 samples were well differentiated (Gleason score < 7), 27 samples were moderately differentiated (Gleason score = 7), and 11 samples were poorly differentiated (Gleason score > 7; Table 1). No significant difference in nuclear ErbB3 was found between different Gleason scores (ANOVA, \( P = 0.086 \)); however, it is important to note that some tissue sections contained several Gleason patterns. Therefore, we reasoned that a whole tissue section may not be appropriate to assess nuclear expression of ErbB3 in relation to the Gleason grade. Consequently, we favored the analysis from tissue cores contained in the tissue array to estimate the correlation between ErbB3 nuclear expression and Gleason grade.

In line with the observations made on tissue sections, nuclear localization of ErbB3 in normal cores from patients with prostate cancer contained in the tissue array was rare (5%, 2 of 43 cores; see Table 4). Statistical analyses confirmed that ErbB3 nuclear localization was significantly more frequent in cancerous tissues compared with normal tissue cores (\( P = 0.048 \), Fisher’s exact test; Table 4). ErbB3 nuclear staining was observed in 20% of PIN cores from patients with cancer, 32% of low Gleason grade cores, 64% of high Gleason grade cores, and in 85.7% of hormone-refractory cores (Table 4). Although there was a clear increase in nuclear ErbB3 localization in hormone-refractory tissue cores compared with low Gleason

![Fig. 2. Expression and subcellular localization of ErbB3 in prostate cancer cell lines. The subcellular localization of ErbB3 (A) was tested by Western blotting in cytoplasmic and nuclear-enriched fractions of these cell lines. Androgen-sensitive cell lines LNCaP and 22Rv1 displayed the highest expression of ErbB3 in the nuclear extracts compared with androgen-insensitive PC-3 and DU145 cells. The purity of nuclear extract fractions was evaluated by the absence of cytoplasmic marker α-tubulin. The expression of ErbB3 tested in whole-cell extracts by Western blot (B) also showed higher expression of ErbB3 in LNCaP and 22Rv1 compared with PC-3 and DU145 cells. The human breast cancer cell line MCF-7 was used as a positive control for ErbB3. The ErbB3/actin ratio was made by Image-Pro-Plus densitometry analysis.

We further attempted to analyze the relation between nuclear localization of ErbB3 and Gleason score among 92 hormone-sensitive tissues obtained by radical prostatectomy. In the 92
grade and high Gleason grade cancer tissue cores, it is important to note that only seven hormone-refractory cores were present on the array (Table 4). There was also a significant increase in the frequency of cores displaying ErbB3 nuclear staining with increasing Gleason score ($P < 0.05$, Fisher’s exact test; Table 4).

### Discussion

Although the molecular events associated with the progression of prostate cancer are complex and are not well established, ErbB receptor signaling has been shown to play an important role in tumor proliferation (6). The present study assessed the expression and subcellular localization of ErbB3 in prostate cancer cell lines and tissues. Although nuclear localization of ErbB receptors has been described in other malignancies (34–38), to our knowledge, this study provides the first evidence of nuclear localization of ErbB3 in prostate cancer cell lines and tissues.

In agreement with previously published studies (13), we observed higher expression of ErbB3 in whole-cell extracts of androgen-sensitive prostate cancer cell lines (LNCaP and 22Rv1) compared with androgen-insensitive cell lines (PC-3 and DU145). Although detectable in all cell lines analyzed, nuclear ErbB3 was more prevalent in androgen receptor (AR)–positive prostate cancer cell lines compared with AR-negative cell lines. This observation may suggest a relationship between AR and expression and nuclear localization of ErbB3. Interestingly, a recent report indicates that the ErbB2/ErbB3 pathway mediates the modulation of AR function (41); it was noted that ErbB2/ErbB3 pathway stabilized AR protein levels and optimized the binding of AR to the promoter regions of androgen-regulated genes (41). Our observations of high nuclear ErbB3 expression in hormone-refractory prostate cancer tissues parallels the fact that AR expression is often up-regulated in hormone-refractory prostate cancer tissues (42, 43). Thus, it is tempting to speculate that nuclear ErbB3 modulates AR expression, or alternatively, that AR activity plays a role in ErbB3 nuclear localization. Future studies targeting the AR and/or ErbB3 in LNCaP or 22Rv1 cell lines may help to clarify some of these issues.

The mechanism of ErbB3 nuclear translocation is still unknown. One hypothesis is that ErbB3 heterodimerizes with EGFR, as the latter has already been observed in the nucleus of prostate cancer tissues (44). However, in the cell lines used here, ErbB3 was not found to heterodimerize with EGFR in unstimulated conditions (14), excluding such a hypothesis to explain the mechanism of ErbB3 nuclear translocation observed here. Interestingly, some studies have shown that in presence of neuregulin1-p1, an ErbB3-binding protein 1 dissociates from ErbB3, translocates to the nucleus, and represses AR transcriptional activity by binding to the AR (45, 46). As our results show that ErbB3 is present in the nucleus in an unphosphorylated form, it would be interesting to determine whether it remains associated with ErbB3-binding protein 1 under these conditions, especially in light of the fact that inhibition of ErbB3 phosphorylation also inhibits the transactivation of the AR (47). These observations indicate a potential role of nuclear ErbB3 in modulating AR activity.

Previous studies have reported membranous and cytoplasmic ErbB3 staining varying from 20% to 95% with no relation to tumor grade (15, 16, 33). In this study, ErbB3 cytoplasmic expression was observed in 100% of prostate cancer tissue samples and in about 90% of cores present on the tissue array. These discrepancies may be due to varying protocols and to the inherent subjectivity of the different scoring systems. Regardless, as was observed by the others, there was no statistically significant correlation between the level of overall ErbB3 expression and histopathologic grade (Gleason score) of prostate tissues in our cohort.

In tissue sections, we observed that nuclear ErbB3 is not frequently present in normal and in BPH tissues, and that its frequency increases as pathologic status worsens (from 45% in hormone sensitive to 100% in hormone refractory). This observation was also confirmed in tissue array cores and was statistically significant ($P < 0.05$), indicating that nuclear ErbB3 may play a role in prostate cancer progression.

On the tissue array, 20% of tissues from PIN cores showed positive nuclear staining for ErbB3, whereas almost no normal cores from prostate cancer patients stained for ErbB3. Clearly, it will be of interest to determine whether nuclear ErbB3 in PIN tissues correlates with eventual progression to malignant prostate cancer. For this purpose, a study comparing ErbB3 status in PIN tissues from patients who eventually develop invasive carcinoma with PIN specimens from patients who remain free for malignancy is planned.

In several cancers, the association between elevated ErbB receptors levels and clinical outcome is controversial. Although one study showed that ErbB2 is overexpressed in prostate cancer compared with BPH (48), ErbB2 overexpression was also found to be unrelated to clinical and pathologic stage (10, 48), whereas other studies observed a correlation with progression to androgen-independent prostate cancer (49, 50). There is similarly conflicting evidence concerning the usefulness of ErbB3 in cancer prognosis. Some studies show an association between the overexpression of ErbB3 with a malignant phenotype in gastric cancer cell lines (51), with tumor growth in carcinoma of the oral mucosa (52) and with tumor proliferation and patient survival in breast carcinoma (53). On the other hand, another study found no correlation between ErbB3 expression and tumor size and concluded that ErbB3 could not be used as a prognostic marker for breast cancer (54). In a similar way, one study showed that membranous and cytoplasmic ErbB3 expression may be helpful to identify oral squamous cell carcinomas of high malignant potential (30), whereas according to another study, ErbB3 was unable to predict patient survival in these cancers (25).

### Table 4. Tissue array: ErbB3 nuclear staining in function of histopathologic grade

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>PIN</th>
<th>LG</th>
<th>HG</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-nuclear</td>
<td>41</td>
<td>20</td>
<td>136</td>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear (%)</td>
<td>2 (5)</td>
<td>5 (20)</td>
<td>64 (32)</td>
<td>74 (64)</td>
<td>6 (85.7)</td>
</tr>
<tr>
<td>Fisher’s exact test, $P$</td>
<td>0.095</td>
<td>0.048</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR</td>
<td>0.002</td>
<td>0.007</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: LG, low Gleason grade; HG, high Gleason grade; HR, hormone refractory.
The ErbB family receptors are widely accepted as tyrosine kinase receptors and are thought to function as signal initiators from the cell membrane. However, in accordance with our study, growing evidence is providing a link between the nuclear localization of ErbB receptors and increased proliferation or tumor development. The nuclear translocation of ErbB receptors was found in different studies in breast, ovarian, glial, and oral cancer cells and tissues (35, 38, 55, 56). For example nuclear ErbB1 and ErbB2 have been observed in breast cancer tissues and cell lines (35, 38). More recently, a report indicates that EGFR nuclear localization is observed in breast cancer tissues and reveals an inverse correlation between high nuclear EGFR and overall survival (34). ErbB3 has been observed in the nucleus of normal and cancerous mammary epithelial cells lines (36), as well as in the nuclei of myelinating glial cells (57). Nuclear localization of ErbB4 was reported in breast cancer (37) and in primary osteosarcoma (58). The nuclear localization of ErbB members supports a new potential role for these receptors as transcription factors, cofactors or coactivators. In fact, EGFR associates with the promoter region of cyclin D in vivo in the MDA-MB-468 breast cancer cell line (35), whereas ErbB2 was found to form a complex at a specific nucleotide sequence of the cyclooxygenase-2 gene promoter in the MCF-7 breast cancer cell line (38). By extrapolation and because ErbB members have similar functional domains, it is possible that ErbB3 may also have a transcription-regulation function. Although hypothetical, an additional possibility is that the nuclear sequestering of ErbB3 receptors may alter the propensity to form ErbB3-containing homodimers or heterodimers at the level of the plasma membrane. This would have the general effect of altering the response to growth factors, favoring pathways that are activated by ErbB family members that remain in the cytoplasmic membrane. Further work will be needed to evaluate each of these possibilities in the case of prostate cancer.

In conclusion, this is the first report of nuclear localization of ErbB3 in prostate cancer cell lines and tissues. Furthermore, nuclear localization correlated with the pathologic status of prostate cancer samples analyzed. Additionally, the correlation of nuclear ErbB3 with tumor grade and particularly the high frequency of ErbB3 nuclear localization in hormone refractory indicates that ErbB3 warrants further studies to understand the critical molecular events associated to the prostate cancer disease progression.

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References

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