Bone Microenvironment and Androgen Status Modulate Subcellular Localization of ErbB3 in Prostate Cancer Cells

Chien-Jui Cheng,1 Xiang-cang Ye,2 Funda Vakar-Lopez,3 Jeri Kim,4 Shi-Ming Tu,4 Dung-Tsa Chen,7 Nora M. Navone,4 Li-Yuan Yu-Lee,6 Sue-Hwa Lin,2,4 and Mickey C-T. Hu5

1Department of Pathology, Taipei Medical University and Hospital, Taipei, Taiwan; Departments of 2Molecular Pathology, 3Pathology, 4Genitourinary Medical Oncology, and 5Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center; 6Departments of Medicine, Molecular and Cellular Biology, and Immunology, and Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, Texas; and 7Biostatistics Division, Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida

Abstract
ErbB-3, an ErB receptor tyrosine kinase, has been implicated in the pathogenesis of several malignancies, including prostate cancer. We found that ErbB-3 expression was up-regulated in prostate cancer cells within lymph node and bone metastases. Despite being a plasma membrane protein, ErbB-3 was also detected in the nuclei of the prostate cancer cells in the metastatic specimens. Because most metastatic specimens were from men who had undergone androgen ablation, we examined the primary tumors from patients who have undergone hormone deprivation therapy and found that a significant fraction of these specimens showed nuclear localization of ErbB3. We thus assessed the effect of androgens and the bone microenvironment on the nuclear translocation of ErbB-3 by using xenograft tumor models generated from bone-derived prostate cancer cell lines, MDA PCa 2b, and PC-3. In subcutaneous tumors, ErbB-3 was predominantly in the membrane/cytoplasm; however, it was present in the nuclei of the tumor cells in the femur. Castration of mice bearing subcutaneous MDA PCa 2b tumors induced a transient nuclear translocation of ErbB-3, with relocation to the membrane/cytoplasm upon tumor recurrence. These findings suggest that the bone microenvironment and androgen status influence the subcellular localization of ErbB-3 in prostate cancer cells. We speculate that nuclear localization of ErbB-3 may aid prostate cancer cell survival during androgen ablation and progression of prostate cancer in bone. (Mol Cancer Res 2007;5(7):675–84)

Introduction
The ErbB family of membrane proteins consists of receptor tyrosine kinases that mediate cell growth and differentiation through the binding of their ligands (1). In response to cognate ligands such as heregulin, ErbB-3 forms heterodimers with ErbB-2, and those heterodimeric ErbB-2/ErbB-3 complexes activate a phosphatidylinositol-3-kinase–dependent signaling pathway (2, 3). Aberrant increases in ErbB-3 expression have been implicated in a variety of malignancies, including prostate cancer. For example, ErbB-3 mRNA levels have been elevated in human mammary tumor cell lines (4), and overexpression or gene amplification of ErbB-3 has been reported in various carcinomas and cancer cell lines (5, 6). Moreover, high ErbB-3 protein expression has been linked with poor prognosis in endometrioid carcinoma of the ovary (7) and with short survival in advanced non–small cell lung carcinoma (8). Several reports indicate that ErbB-3 expression increases in parallel with the malignancy of prostate cells, implicating ErbB-3 in the progression of prostate cancer (9-12). However, information is limited on the expression of ErbB-3 in the metastasis of prostate cancer.

For any type of cancer, the microenvironment is critical for the metastatic progression of cancer cells to distant sites (13). The environmental influence is especially apparent in prostate cancer in that bone is by far the most common site of metastasis (14). The propensity of prostate cancer to metastasize to bone suggests that interactions between the metastatic prostate cancer cells and the tumor microenvironment are important in the progression of prostate cancer (15-18). Delineating the mechanisms involved in promoting metastatic prostate tumor progression in the bone will lead to better treatment strategies for bone metastasis.

In this study, we investigated the role of ErbB-3 in the metastatic progression of prostate cancer. We found expression of ErbB-3 to be up-regulated in metastases relative to expression in primary tumor. We further found that ErbB-3 in metastases in bone was largely in the nuclei of the metastatic
prostate cancer cells. Because metastasis to bone often occurs after prostate cancer becomes androgen independent, we used mouse xenograft models to investigate the effect of androgens and the bone microenvironment on the nuclear translocation of ErbB3 in prostate cancer. Our findings suggest that the cellular localization of ErbB-3 in prostate cancer cells is a dynamic process that can be influenced by the tumor microenvironment and androgen status. Possibly, nuclear translocation of ErbB-3 is one of mechanisms involved in the androgen-independent progression and metastasis of prostate cancer to bone.

Results

ErbB-3 Expression Patterns in Prostate Cancer Specimens

To examine the pattern of expression of ErbB-3 in prostate cancer specimens, we used antibodies against the intracellular domain (ICD) of ErbB-3 to stain specimens from patients with localized or disseminated metastatic prostate cancer (Table 1). We found that epithelial cells in 50 of 52 prostate cancer specimens from patients with primary localized prostate cancer expressed very low or undetectable levels of ErbB-3 (Fig. 1A). In contrast, positive staining of ErbB3 was more common in prostate cancer cells that had metastasized to lymph nodes [12 of 19 (63%)] or to bone [12 of 26 (46%); Fig. 1A; Table 2]. The differences in ErbB-3 staining were significant for both lymph node metastases and bone metastases relative to ErbB-3 staining in the primary prostate cancer specimens (P < 0.001 for both). Moreover, more cells in the metastases were positively stained for ErbB-3 than in the primary tumor; the mean percentage of positive cells was 96% for lymph node metastases and 87% for bone metastases, compared with 7.5% for primary tumor (P = 0.002 for lymph node versus primary and P = 0.008 for bone versus primary). Thus, both the frequency of ErbB-3 positivity and the number of ErbB-3–positive cases, showed nuclear localization of ErbB-3 staining (Table 2). Examples of nuclear and mixed nuclear and membrane/cytoplasmic staining in a lymph node specimen are shown in Fig. 1B.

Of the 12 bone metastasis specimens that showed ErbB-3 staining, eight cases showed exclusively or predominantly nuclear staining (P < 0.001 versus primary tumor), and four cases showed exclusively or predominantly cytoplasmic ErbB-3 staining (Table 2). Examples of nuclear and mixed nuclear and membrane/cytoplasmic staining in a bone metastasis specimen are shown in Fig. 1C.

In summary, among 45 human prostate cancer specimens from lymph node and bone metastases, 24 cases showed detectable ErbB-3, and 12 of them, representing 50% of the ErbB-3–positive cases, showed nuclear localization of ErbB-3 (Table 2). These observations suggest that expression of ErbB-3 is not only elevated but also the ErbB3 protein is increasingly translocated from the membrane/cytoplasm to the nuclei of cancer cells that metastasized to the lymph nodes or bone.

Androgen Status and Nuclear ErbB-3 in Metastatic Prostate Cancer Specimens

Because metastatic progression of prostate cancer often follows the development of androgen-independent disease, we examined ErbB-3 localization in terms of the androgen status of the specimen donors (Table 1). Among four lymph node specimens that showed positivity for nuclear ErbB-3 staining (Table 2), three of them were from men who had undergone androgen ablation, whereas all eight bone metastasis specimens positive for nuclear ErbB-3 (Table 2) were from the donors under androgen ablation, suggesting that nuclear localization of ErbB-3 may be related to androgen status of patients.

Nuclear Localization of ErbB-3 in Primary Tumors of Patients Who Had Undergone Androgen Deprivation Therapy

Although it would be informative to examine the expression and subcellular localization of ErbB-3 in bone metastasis.

Table 1. Clinical and Pathologic Characteristics of Human Prostate Cancer Samples

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>Specimen Source</th>
<th>Total No. Samples</th>
<th>Gleason Score (No. of Samples)</th>
<th>Androgen Ablation (No. of Patient Samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor</td>
<td>Radical prostatectomy</td>
<td>20</td>
<td>6 (5)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 (7)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>8 (2)</td>
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<td>9 (6)</td>
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<td>6 (14)</td>
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<td>9 (9)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 (3)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>Proximal and distal</td>
<td>19</td>
<td>N/A</td>
<td>14</td>
</tr>
<tr>
<td>Bone metastases</td>
<td>Vertebral and long bone</td>
<td>26</td>
<td>N/A</td>
<td>25</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, not applicable.
specimens from patients that have not undergone androgen ablation, we were not able to obtain such specimens. We thus examined 14 primary prostate tumors from patients who had undergone androgen deprivation therapy before prostectomy. We found that 7 of these 14 specimens showed positive staining of ErbB-3. This frequency is much higher than that of the primary prostate cancer specimens from patients without androgen deprivation therapy (Table 2). Of the seven specimens that showed positive staining of ErbB-3, three cases showed exclusively or predominantly nuclear staining; whereas other four cases showed exclusively or predominantly cytoplasmic ErbB-3 staining. These observations imply that aberrant ErbB3 expression and translocation may be influenced by factors under androgen ablation.

**Nuclear ErbB-3 in Prostate Cancer Cells Contains Both Extracellular Domain and ICD**

To examine whether the nuclear ErbB-3 protein contains an extracellular domain (ECD), the specimen sections that showed positive staining of ErbB3-ICD in nucleus were subjected to further examination by immunostaining with an ErbB3-ECD–specific antibody. Consecutive sections from the same specimens were stained to directly compare the distribution of extracellular domain versus ICD of ErbB-3. In lymph node and bone metastasis specimens, both antibodies produced strong nuclear ErbB-3 staining (Fig. 2), indicating that the Erb-3 present in the nuclei of metastatic prostate cancer cells contained both the ECD and ICD of ErbB-3.

**Subcellular Localization of ErbB-3 in the Prostate Cancer Cells Cultured in vitro**

Although the nuclear localization of ErbB-3 was found in prostate cancer tumor specimens, it was not known whether this also occurs in prostate cancer cells cultured in vitro. We did biochemical analyses and immunostaining of ErbB3 in two prostate cancer cell lines, MDA PCa 2b (19) and PC-3 (20), both of which were originally derived from bone metastases from men with advanced prostate cancer. By biochemical fractionation, immunoprecipitation, and Western blotting to detect ErbB-3, we found that ErbB-3 was expressed in both cell lines, mainly in the membrane/cytoplasmic fraction (Fig. 3A). Immunostaining of these prostate cancer cells with the ErbB3-ICD antibody showed that ErbB-3 was mainly in the membrane/cytoplasm of these cells (Fig. 3B and C). This is consistent with the cell fractionation findings. Moreover, the subcellular localization of ErbB3 in MDA PCa 2b and PC-3 cells was not affected by culture conditions with or without supplement of dehydrotestosterone (data not shown), suggesting that androgen may have an indirect effect on ErbB3 translocation probably through intermediate factors in tumor microenvironment.

**Table 2. ErbB-3 Staining in Human Prostate Cancer Specimens**

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>ErbB-3 Positive</th>
<th>Patterns of ErbB-3 Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%* p↑</td>
<td>N or N &gt; C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%* p↑</td>
</tr>
<tr>
<td>Primary prostate cancer tumors (n = 52)</td>
<td>2 (4%) —</td>
<td>0 (0%) —</td>
</tr>
<tr>
<td>Lymph node metastases (n = 19)</td>
<td>12 (63%) &lt;0.001</td>
<td>4 (21%) 0.004</td>
</tr>
<tr>
<td>Bone metastases (n = 26)</td>
<td>12 (46%) &lt;0.001</td>
<td>8 (31%) &lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: N, nuclear; C, cytoplasmic.

*Percent of total cases.
†Fisher’s exact test.
Nuclear Localization of ErbB-3 in the Bone of Mouse Xenograft Model

Because the in vivo bidirectional interactions between the tumor cells and the bone microenvironment are critical for the development of bone metastases (21, 22), we next investigated the expression and subcellular localization of ErbB-3 in mouse xenograft models of prostate cancer. We found that in tumors formed by both MDA PCa 2b and PC-3 cells implanted at subcutaneous sites, ErbB-3 was present mainly in the cell membrane/cytoplasm (Fig. 4A). In contrast, in tumors from these prostate cancer cells implanted in mouse femurs, ErbB-3 was highly concentrated in the nuclei (Fig. 4B). These in vivo findings suggest that the bone microenvironment could influence the nuclear localization of ErbB-3 in prostate cancer tumors growing in bone.

To examine whether both the ECD and ICD of ErbB-3 are present in the nuclei of MDA PCa 2b tumor cells grown in mouse bone, immunofluorescence staining was used to colocalize ErbB3-ECD and ICD on the same slide. As shown in Fig. 4C, both the ECD- and ICD-specific antibodies produced strong nuclear ErbB-3 staining, indicating that intact ErbB-3 protein is likely involved in nuclear translocation. This observation is consistent with those found in human specimens (Fig. 2).

Translocation of ErbB-3 in Response to Androgen Status in Mouse

With the mouse xenograft model, we also assessed the effect of androgen deprivation on the subcellular localization of ErbB-3 in MDA PCa 2b cells, which is one of the few prostate cancer cell lines responsive to androgen deprivation in vivo (19). In the experiments, mice bearing subcutaneous MDA PCa 2b tumors were castrated and tumors were collected at various intervals thereafter. Significant decreases in tumor size and serum prostate-specific antigen levels were evident at 2 weeks after castration (Fig. 5A). With regard to subcellular location of ErbB-3 within the tumor cells, we did immunostaining of tumors from both intact and castrated mice. Results showed that the MDA PCa 2b cells in the tumors from intact mice contained exclusively the membrane/cytoplasmic ErbB-3, whereas the cells of tumors from castrated mice contained predominantly nuclear ErbB-3 at 1 and 2 weeks after castration (Fig. 5B). Nuclear translocation of ErbB-3 in the MDA prostate cancer 2b tumor cells seemed to correspond with temporary arrest of tumor growth under androgen ablation (Fig. 5A and B). These findings suggest that depletion of androgens in vivo may change the tumor microenvironment and lead to the nuclear translocation of ErbB-3 in the prostate cancer tumor cells.

Considering that androgen-independent progression of prostate cancer often occurs long after androgen ablation, we then examined the subcellular localization of ErbB-3 in the MDA PCa 2b tumors collected at 15 weeks after castration (i.e., after the development of androgen-independent disease). Result showed that in these tumors resuming aggressive growth, ErbB-3 had been relocalized mostly to the membrane/cytoplasm of the prostate cancer cells (Fig. 5B). We further did Ki-67 immunostaining on the MDA prostate cancer 2b tumors from intact and castrated mice. As shown in Fig. 5C, there is a significant decrease in Ki-67 staining in tumors at 1 and 2 weeks after castration, whereas the Ki-67 staining significantly increased at 15 weeks after castration (Fig. 5C). These observations suggest that nuclear localization of ErbB-3 correlates with the castration-induced inhibition of cell proliferation of MDA prostate cancer 2b tumors.

FIGURE 2. Immunohistochemical staining of ErbB-3 with antibodies specific to the extracellular domain versus the cytoplasmic domain of ErbB3. Anti-ErbB3 antibodies that recognize either the ICD (RTJ.2) or the extracellular domain (Ab-10) were used to detect ErbB3 in prostate cancer lymph node specimens (A and B) and bone metastasis specimens (C and D). Original magnification, x200.
Together, these observations suggest that the translocation of ErbB-3 within the tumor cells may be a dynamic process in response to tumor microenvironmental changes, which could be triggered by the tumor-host interactions (such as in bone) or induced by androgen ablation. Nuclear translocation of ErbB-3 may reflect an adaptive reaction of tumor cells for different growth modes.

**Discussion**

We report here a novel correlation between subcellular location of ErbB-3 in prostate cancer metastases versus its location in primary tumors and our exploration of possible causes for this difference. We found that ErbB-3 expression was up-regulated in metastases (relative to primary tumors) and that ErbB-3 was located in the nucleus of prostate cancer cells in about half of the ErbB-3-positive human prostate cancer specimens tested. We then showed, using bone-derived prostate cancer cell lines in mouse xenograft models, that nuclear localization of ErbB-3 was induced by the bone microenvironment and that the ErbB-3 translocation was influenced by androgen status. Our findings raise the interesting possibility that nuclear translocation of ErbB-3 may be one of the cellular alterations involved in the androgen-independent progression and metastasis of prostate cancer to bone.

The finding that ErbB-3 protein was located in the nuclei of cancer cells in prostate cancer specimens was unexpected and raises questions regarding the roles of ErbB-3 in the nucleus. Our observations from animal models suggest that ErbB3 nuclear localization is probably involved in growth arrest or survival of tumor cell in certain microenvironment: ErbB-3, a partner with ErbB2/Her2 normally at transmembrane signaling, had shifted from the membrane/cytoplasm to the nucleus in subcutaneous tumors within 2 weeks after androgen ablation (Fig. 5). Once the tumor cells overcame the effects of androgen ablation, ErbB3 reverted to the membrane/cytoplasm localization, suggesting that nuclear ErbB3 perhaps was no longer necessary for androgen-independent tumor progression. Consistent with this possibility, we observed that nuclear localization of ErbB-3 correlated with a decrease in cell proliferation, as reflected by Ki-67 staining, after androgen ablation. The ability of cancer cells to survive in distant organ sites is a critical step in cancer metastasis (23). It is tempting to speculate that nuclear ErbB-3 may be necessary for prostate cancer cells to survive initially in a metastasis site, but once the cells overcome the initial challenge from the new environment, ErbB-3 reverts to the membrane for cell entering a rapid growth mode. Mechanistically, however, it is not clear whether nuclear localization of ErbB-3 is associated with growth arrest or survival of prostate cancer cells.

One of potential mechanisms by which nuclear ErbB-3 could affect cell survival or induce growth arrest is through regulating growth-related transcriptional activity. The nuclear functions of other ErbB receptors have been attributed mainly to their ability to act as transcriptional regulators (24-32). For example, Lin et al. (24) showed that nuclear ErbB-1 acts as a transcriptional regulator that stimulates genes required for cell proliferation (e.g., cyclin D1). Nuclear ErbB-3 has been shown to regulate the expression of the cyclooxygenase-2 gene in human breast cancer cell lines (28). Similarly, Komuro et al. (29) showed that the cytoplasmic domain of ErbB-4 associates with the coactivator Yes-associated protein in the nucleus to regulate gene transcription. Thus, we speculate that nuclear ErbB-3 may act as a transcriptional regulator to modulate the expression of genes involved in cell survival or proliferation. Interestingly, several transcription factors have been found to associate with ErbB-3 in yeast two-hybrid approaches, including p23/p198 protein (also known as Ebp1; ref. 33).
early growth response-1 (34), and the zinc finger protein ZNF207 (34). Yoo, Zhang, and others showed that treating AU565 breast cancer cells with the ErbB-3 ligand heregulin resulted in dissociation of Ebp1 from ErbB-3 and subsequent translocation of Ebp1 to the nucleus, where it suppressed androgen receptor-mediated gene transcription (35-37). Whether Ebp1 can interact with ErbB-3 in the nucleus and whether its transcriptional activity is regulated by nuclear ErbB-3 remain to be studied.

The mechanism by which ErbB-3 is internalized to the nucleus is not clear. Different mechanisms have been found for each ErbB family protein. In the case of ErbB-4, binding with ligand heregulin or activation by protein kinase C through 12-O-tetradecanoyl phorbol-13-acetate leads to the cleavage of the ErbB-4 ectodomain by a metalloprotease (38-40). Subsequent cleavage by γ-secretase releases the ErbB-4 ICD from the membrane and facilitates its translocation to the nucleus (41). Thus, only the cytoplasmic domain of ErbB-4 is translocated into the nucleus. In contrast, Lin et al. (24) and Xie et al. (26) showed that the entire ErbB-1 and ErbB-2 proteins were translocated into the nucleus. Similarly, Offerdinger et al. (42) reported that both extracellular and cytoplasmic domains of ErbB-3 were present in the nuclei of several breast cancer cell lines. Our findings here also showed that both the extracellular and cytoplasmic domains of ErbB-3 translocate into the nucleus. The mechanism by which translocation takes place is unknown but may involve endocytosis and the nuclear pore complex, as was proposed to explain the nuclear translocation of ErbB-1 (43) and ErbB-2 (44).

Nuclear translocation of ErbB-3 is apparently a highly regulated event in prostate cancer, as it was observed in only a subset of tumor specimens from men with advanced prostate cancer. Identification of factors that regulate this process will shed light on its the physiologic and pathologic significance. Our finding that ErbB-3 was localized in the nuclei of prostate cancer cells in bone (Fig. 4) suggests that factors present in the bone microenvironment may regulate the nuclear translocation of ErbB-3. However, in vitro treatment of MDA PCa 2b and PC-3 cells with 10% human bone marrow supernatant in culture medium did not cause nuclear translocation of ErbB-3 in these cells (data not shown), suggesting that the regulatory mechanism in vivo is complex. ErbB-3 receptor cognate
ligands such as heregulin are possible stromal factors involved in the regulation of ErbB-3 nuclear translocation. However, treatment of MDA PCa 2b and PC-3 cells with heregulin-β did not cause nuclear translocation of ErbB-3 in these cells (data not shown). In bone, many factors are secreted by osteoblasts or osteoclasts, which are the major bone stromal cells. Some of these factors may be regulated directly or indirectly by androgens, as nuclear translocation of ErbB-3 takes place during the androgen ablation. Induction of osteoblastic and, to a lesser extent, osteolytic lesions are frequently observed in patients with bone metastasis from prostate cancer and this was attributed to the specific interactions between prostate cancer cells and the bone microenvironment (45-47). However, nuclear localization of ErbB-3 may not be related to the prostate cancer–induced osteoblastic or osteolytic response as it occurs both in the tumors derived from the bone-forming MDA PCa 2b cells and the bone-lysing PC-3 cells (Fig. 4).

While this work was in progress, Koumakpayi et al. (12) reported their findings on the nuclear localization of ErbB-3 in prostate cancer; specifically, in primary prostate tumor specimens, nuclear ErbB-3 was detected more often in hormone-refractory tissues than in hormone-sensitive tissues. Our results are in general agreement with their findings and further extend these observations to prostate cancer metastases in lymph nodes and bone. Whereas Koumakpayi et al. (12) found that 100% of hormone-refractory prostate samples stained positive for ErbB-3 in the nucleus, we found nuclear ErbB-3 in 43% of ErbB-3–positive specimens from patients who had hormone deprivation therapy and 67% of ErbB-3–positive prostate cancer in bone. This difference in the prevalence between these two studies may reflect the dynamic nature of ErbB-3 nuclear localization, which seems to depend on the duration of androgen ablation, as shown by our use of xenograft model of MDA PCa 2b cell line. In our study, only very few primary prostate tumors expressed ErbB-3 at all (2 of 52 cases), and in those cases, only 7.5% of the prostate cancer cells expressed ErbB-3, which was largely in the membrane/cytoplasm. In contrast, Koumakpayi et al. (12) observed ErbB-3 cytoplasmic expression in 90% to 100% of the primary prostate cancer tumor tissues examined. Previous studies by others, on the other hand, indicated that the

FIGURE 5. Localization of ErbB-3 in subcutaneous tumors generated in intact and castrated mice. For generating xenograft tumors, MDA PCa 2b cells were injected subcutaneously into nude mice. Mice were castrated when tumors reached ~ 500 mm³. A. Tumor sizes were monitored weekly. Left, points, average tumor size of six mice observed over 15 wk after castration; bars, SE. Blood prostate-specific antigen levels were monitored biweekly. Right, points, average prostate-specific antigen (PSA) levels of six mice observed over 15 wk after castration; bars, SE. *, P < 0.05 versus tumor size or prostate-specific antigen at day 1. B. Tumors were collected at the indicated periods after castration. Androgen-independent tumors were collected at 15 wk after castration. Tumor specimens were immunostained with anti-ErbB3 antibody RTJ2. C. Tumor specimens were immunostained with anti–Ki-67 antibody. Original magnification, ×400.
Incidence of membranous/cytoplasmic ErbB-3 staining varies from 14% to 95% (9-11, 48). The discrepancies among these findings may reflect variations in protocols and the inherent subjectivity of the different scoring systems. Regardless, our analysis of clinical samples provides a significant link between nuclear translocation of ErbB-3 and prostate cancer metastasis, in both lymph nodes and bone. Our use of two xenograft models and two prostate cancer cell lines further substantiates these observations from clinical samples.

In conclusion, we found ErbB-3 in the nucleus in specimens of metastatic lesions from men with advanced prostate cancer. Our mouse xenograft studies suggest that the nuclear localization of ErbB-3 may be regulated, at least in part, by factors present in the bone microenvironment and by androgen status, two major elements involved in the metastatic progression of prostate cancer. Our results raise the interesting possibility that nuclear ErbB-3 may be involved in the progression of prostate cancer in bone after androgen-ablation therapy.

Materials and Methods

Immunostaining of Prostate Cancer Specimens

Formalin-fixed, paraffin-embedded tissue samples representing a spectrum of localized and metastatic prostate cancer, including radical or transurethral prostatectomy specimens, lymph nodes, and bone specimens with prostate cancer metastases, were selected from a prostate cancer tissue bank (supported by a Specialized Program of Research Excellence award to The University of Texas M.D. Anderson Cancer Center). Clinical and pathologic characteristics are shown in Table 1.

A mouse monoclonal antibody against the cytoplasmic domain of ErbB-3 (RTJ.2; Santa Cruz Biotechnology) and a polyclonal antibody against the extracelluar domain of ErbB-3 (Ab-10; NeoMarker/Lab Vision) were used for immunohistochemical analysis as follows. Four-micrometer-thick sections were dewaxed with xylene, rehydrated in graded concentrations of alcohol, treated with 3% H2O2 in methanol for 15 min, washed with PBS, blocked with normal horse serum for 30 min, and incubated at 4°C overnight with RTJ.2 (2 μg/mL) and Ab-10 (5 μg/mL). Antibody binding was detected by using a labeled streptavidin-biotin kit with 3,3′-diaminobenzidine as the chromogen (DAKO). Hematoxylin was used as the counterstain.

The scoring system derived to describe the relative expression of ErbB-3 in the nucleus and membrane/cytoplasm of the prostate cancer specimens was as follows. Based on the staining patterns in the sections prepared as described above and observed by microscopy, expression of ErbB-3 in tumor cells was considered to be in the nucleus only (N), in the membrane/cytoplasm only (C), in both the membrane/cytoplasm and nucleus, with nuclear staining being more prominent (C > N), and in both membrane/cytoplasm and nucleus, with nuclear staining being more predominant (N > C). For statistical purposes, both the C and C > N cases were considered to represent membrane/cytoplasm localization, and the N and N > C cases nuclear localization. All staining data were reviewed independently by two pathologists; differences in scoring were resolved by consensus after concurrent review by both pathologists.

Differences in the proportions of specimens expressing ErbB-3 in primary prostate cancer tumors versus lymph node or bone metastases were assessed with Fisher’s exact test. In the analysis of percent ErbB-3–positive tumor cells, arcsine-root transformation was used to improve normality (49). Two-sample t tests were then used to test the difference of the transformed percentage of ErbB-3–positive tumor cells between the primary prostate cancer tumor versus the lymph node or bone metastases.

Nuclear and Cytoplasmic Fractionation and Analysis

The prostate cancer cell line PC-3 was purchased from the American Type Culture Collection (20). MDA PCa 2b cells, generated from a bone metastasis from a man with prostate cancer, were obtained from the Department of Genitourinary Medical Oncology at M.D. Anderson Cancer Center (19). PC-3 cells (maintained in DMEM with 10% FCS) and MDA PCa 2b cells [maintained in BRFF-HPCI medium (AthenaES, Baltimore, MD) with 20% FCS] were seeded in 100-mm plates and grown to subconfluence.

The nuclear and plasma membrane/cytoplasm fractions of prostate cancer cells, MDA PCa 2b, and PC-3 cells (1 × 10⁶ each) were separated by an NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce) as described by the manufacturer. The fractions were resuspended in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 0.5% deoxycholate, 1% NP40], and insoluble material was removed by centrifugation. The supernatants were incubated with an anti–ErbB-3 antibody (2F12; Neomarker) for 16 h at 4°C. Immune complexes were collected after incubation for 2 h at room temperature with protein G-agarose (Amersham), separated on 12% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with an anti–ErbB-3 antibody (C-17, Santa Cruz Biotechnology). Signal was detected with the Amersham enhanced chemiluminescence system.

Immunostaining of Prostate Cancer Cell Lines

For ErbB-3 immunostaining of cultured prostate cancer cell lines, cells were seeded on coverslips in a six-well plate and grown to 80% confluence. Cells were then washed with PBS, fixed in 2% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.1% Triton X-100 in PBS (pH 7.5) for 30 min at room temperature. Cells were then blocked in 5% normal horse serum in PBS for 30 min at room temperature, and incubated with RTJ.2 (2 μg/mL in 5% normal horse serum) overnight at 4°C. The next morning, the cells were washed several times with PBS, incubated with FITC-conjugated goat anti-mouse antibody (1:100 in 5% horse serum/PBS) for 30 min at room temperature, mounted with Vectashield and 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), and evaluated under a Leica TCS-SP5 spectral laser scanning confocal microscope.

Subcutaneous and Intramuscular Injection of Prostate Cancer Cells

All procedures involving animals were done in compliance with the guidelines of M. D. Anderson Institutional Animal Research.
Care and Use Committee and the NIH. To generate subcutaneous tumors, PC-3 cells (1 × 10^5) or MDA PCa 2b cells (4 × 10^5) were injected subcutaneously into five 6-week-old male nude mice (Harlan Sprague-Dawley). Tumor development in each animal was measured with calipers twice weekly, and tumor volume was calculated as length × width × height × 0.5236 (the formula of an ellipsoid). When tumors reached 500 mm^3, the mice were killed by cervical dislocation under anesthesia and the tumors were excised, fixed in formalin, and embedded in paraffin for immunostaining.

To study tumor growth in bone, PC-3 cells (3 × 10^5) or MDA PCa 2b cells (3 × 10^5) were injected intrafemorally into five 6-week-old male severe combined immunodeficient mice. Bone injections were done under anesthesia. A 32-gauge needle (Hamilton) was inserted 3 mm into the distal end of the right femur using a drilling motion, and 3 μL of the cell suspension was injected. The left femur was injected with 3 μL of saline. Tumor progression was monitored every 2 weeks by radiography and palpation under anesthesia. Animals were killed 8 weeks after injection, when obvious tumor growth had developed. After the mice were killed, both the tumor-bearing and the sham-injected legs were harvested. All tumors were fixed in formalin, decalcified, and then embedded in paraffin for immunostaining.

Androgen Ablation in Mice Bearing Subcutaneous Tumors

For generating xenograft tumors, MDA PCa 2b cells (4 × 10^5) were injected subcutaneously into 6- to 8-week-old male nude mice (n = 21) and tumor development was followed as described above. Surgical castration was done when tumor volume reached ~ 500 mm^3. Five mice were killed before castration (control); five mice were killed at 1 week after castration; and another five were killed at 2 weeks after castration. Tumor volume and serum prostate-specific antigen levels were measured weekly and biweekly, respectively, after androgen ablation. Recurrent tumors were removed from the remaining six mice at about the 15th week after castration. All tumors were fixed in formalin and embedded in paraffin for immunohistologic analyses. Immunostaining with Ki-67 antibody (clone MIB-1, DakoCytomation) was done according to manufacturer’s instruction.

References

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