Human Epidermal Receptor-2 Expression in Prostate Cancer

Benjamin F. Calvo, Aaron M. Levine, Mavie Marcos, Qu F. Collins, Mary V. Iacocca, Laura S. Caskey, Christopher W. Gregory, Yuhua Lin, Young E. Whang, H. Shelton Earp, and James L. Mohler

Department of Surgery, Divisions of Surgical Oncology [B. F. C.] and Urology [A. M. L., M. M., Q. F. C., C. W. G., J. L. M.], Department of Pathology and Laboratory Medicine [M. V. I., J. L. M.], Department of Pediatrics, Laboratories for Reproductive Biology [C. W. G.], Department of Medicine, Division of Hematology/Oncology [Y. E. W.], and the University of North Carolina–Lineberger Comprehensive Cancer Center [B. F. C., L. S. C., Y. L., Y. E. W., H. S. E., J. L. M.], University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

ABSTRACT

Purpose: Efforts to conclusively establish that human epidermal receptor (HER)-2 overexpression is important to androgen-dependent carcinoma of the prostate (AD-CaP) or to progression to androgen independence (AI-CaP) have failed because of variability in tissue procurement, antibodies, immunostaining procedures, and assessment methods. However, because some in vitro and animal model data correlate HER-2 overexpression with progression to androgen independence, trials of agents that target the HER-2 receptor are under way. To clarify human tumor findings, we studied HER-2 expression at the gene (DNA), mRNA, and protein levels in well-characterized CaP specimens.

Experimental Design: Fifty AD-CaP and 25 AI-CaP specimens from similar numbers of Caucasian and African Americans were immunostained for HER-2 receptor. HER-2 mRNA levels were measured using real-time fluorescence quantitative PCR in patients for whom frozen specimens were available. HER-2 amplification was evaluated using fluorescent in situ hybridization.

Results: HER-2 receptor immunostained in 52% of androgen-dependent and one (4%) androgen-independent tumor. HER-2 immunostaining was not related to age, race, serum prostate-specific antigen levels, or pathologic stage and Gleason grade. HER-2 overexpression was not detected in AI-CaP at the mRNA or gene level. Mean HER-2 mRNA expression was higher (P < 0.05) in AD-CaP than AI-CaP (22,080 versus 15,496 HER-2 copies). HER-2 was not amplified in any of 20 AD-CaP or 19 AI-CaP specimens.

Conclusions: HER-2 protein and message overexpression and HER-2 amplification were not found in AI-CaP.

INTRODUCTION

CaP is the most common malignant neoplasm afflicting American men and the second leading cause of cancer mortality. In the United States in 2002, an estimated 189,000 new cases will be diagnosed and 30,200 men will die from CaP (1). If advanced at diagnosis or upon failure of curative therapy, CaP may be palliated because of its dependence upon androgens for growth. Androgen deprivation therapy activates an apoptotic cascade in CaP cells that results in irreversible cellular damage and death (1). Most patients treated with androgen deprivation therapy demonstrate a decline in PSA that is associated with significant clinical responses; however, almost all patients eventually develop AI-CaP from which death ensues (2).

Factors responsible for progression of CaP to androgen independence remain poorly understood (3). In vivo effects of androgen deprivation therapy were studied by castrating mice bearing androgen-dependent human CWR22 CaP xenografts. IHC and Western blot analysis of these tumors revealed a transient increase in HER-2 and transforming growth factor α protein levels on day 7 after castration. Similar immunoblot analysis revealed that androgen-independent sublines of the androgen-dependent LAPC-4 human CaP xenograft expressed higher levels of HER-2 than their progenitors (4). In addition, overexpression of HER-2 in the androgen-sensitive LNCaP human CaP cell line rescued these cells from growth arrest induced by androgen deprivation in vivo and enhanced tumor growth in castrated hosts in vivo (4). Yeh et al. (5) provided a possible mechanistic explanation by showing that HER-2 overexpression enhanced interaction between androgen receptor and androgen receptor coactivators, which increased PSA levels. In aggregate, these findings suggest a role for aberrant expression of HER-2 in prostate carcinogenesis as well as progression to androgen-independent growth.

HER-2 (c-erbB-2) encodes the 185-kDa transmembrane tyrosine kinase receptor first identified as a homologue of the epidermal growth factor receptor gene (6). Its murine counterpart, neu, was isolated from ethyl nitosurea-induced rat neuroblastomas and was activated by a valine 659 to glutamine point...
mutation within the transmembrane domain (7, 8). HER-2 is now recognized as a member of the epidermal growth factor receptor family of transmembrane receptors encoded by erbB-1 or HER-1 (8–11), erbB-3/HER-3 (12, 13), and erbB-4/HER-4 (tyro2; Ref. 14). HER-1, HER-2, and HER-4 are most related in their tyrosine kinase domains with 80% similarity (15). With the exception of HER-3, which is kinase inactive, activation of the receptors by dimerization leads to autophosphorylation of intracellular tyrosine residues that, in turn, stimulate substrate binding and initiation of specific signaling cascades.

Ligands for this receptor family include EGF, transforming growth factor α, heparin binding EGF, amphiregulin, epiregulin, betacellulin, and neuregulins 1, 2, 3, and 4 (8, 16–18). Transmembrane precursors of the ligands are cleaved into active soluble forms or alternatively function as noncleaved membrane-bound hormones in a juxtacrine or paracrine fashion (19). The receptors assemble as homodimers or heterodimers. The heterodimers exhibited higher ligand affinity as well as more potent biological activity than homodimers upon ligand binding; for example, HER-2-HER-3 heterodimers exhibit strong proliferative activity (20, 21).

Many mechanistic explanations have been advanced to explain the importance of HER-2 overexpression, especially for breast carcinomas. Despite its inability to bind ligand, overexpressed HER-2 can exhibit constitutive tyrosine kinase activity (22). In addition, as HER-2 is the preferred partner of ligand-driven HER-1, HER-3, or HER-4 heterodimers, any ligand stimulation can be enhanced by overexpressed HER-2 (23, 24). Finally, HER-2 heterodimers exhibit augmented signaling, compared with homodimers, through multiple mechanisms: overexpression of HER-2 inhibits its own down-regulation and that of HER-1 resulting in increased signaling (25); HER-2 enhances signaling of EGF and neuregulin ligands by slowing their release from their respective receptors (HER-1 and either HER-3 or HER-4; Ref. 26); and HER-2 can initiate an extremely strong mitogenic signal by HER-3 (27).

Given the therapeutic potential of HER-2, a recombinant humanized anti-HER-2 monoclonal antibody (rhuMAB HER-2; trastuzumab) was developed from a murine monoclonal antibody 4D5 (28). Cobleigh et al. (29) reported that the anti-HER-2 monoclonal antibody, Herceptin (Genentech, San Francisco CA), administered as a single agent, produced durable objective responses in women with HER-2 overexpressing breast carcinoma metastases. Furthermore, when used with anthracycline-based chemotherapy, Herceptin increased the clinical response in patients with metastatic breast cancer that overexpressed HER-2 (30).

The excitement generated by these breast cancer trials has been translated into trials using Herceptin to treat advanced CaP. However, studies of HER-2 protein expression in clinical CaP report conflicting results and have failed to establish clearly that HER-2 overexpression is important in CaP progression (31–45). Specific evaluation of HER-2 receptor levels in AI-CaP has been reported by two other groups of investigators (46, 47). In addition, African Americans have both a higher incidence of clinically significant CaP and a higher mortality rate from CaP than Caucasian Americans (44), yet racial differences in HER-2 expression have not been studied.

As IHC data remain inconclusive, we studied HER-2 expression at the gene, message, and protein levels in clinical specimens of CaP. We used the Dako IHC methods, which are now standard, and a FISH assay validated in a multi-institutional Cancer and Leukemia Group B study of breast cancer patients. Highly sensitive real-time fluorescent quantitative PCR was used to measure HER-2 mRNA levels.

**MATERIALS AND METHODS**

HER-2 expression was measured at the gene copy number (DNA), mRNA and protein level in tissue samples of AD-CaP and matched benign prostate, as well as a second group of tissue samples of Al-CaP.

**Tissue Samples.** Fifty formalin-fixed and paraffin-embedded archived specimens of AD-CaP were obtained from 25 Caucasian and 25 African Americans treated by radical prostatectomy for clinically localized disease. Twenty-five archived specimens of Al-CaP were obtained from 10 African Americans and 15 Caucasians. AI-CaP specimens were obtained when patients suffered urinary retention because of locally recurrent CaP appreciated by digital rectal exam and biochemically by rising PSA (123 ± 275 ng/ml) at long intervals after beginning androgen deprivation therapy. Al-CaP was poorly differentiated (Gleason sum grade 9.2 ± 0.7). Of the 75 patients, 29 (19 AD-CaP and 13 Al-CaP) had fresh tissue collected at the time of radical or transurethral prostatectomy. RNA degradation by prostatic nucleases in these fresh tissue samples was minimized in two ways. First, the method of radical prostatectomy was altered to leave the vascular supply to the prostate intact until just before specimen removal. Second, the excised prostate was inked and incised by the urological surgeon. Specimens were processed and snap-frozen immediately in liquid nitrogen. Al-CaP specimens were obtained by transurethral resection and snap-frozen immediately in liquid nitrogen. These methods for prostate tissue procurement have been used since 1988 and prevent RNA degradation that occurs after 20 min of warm ischemia (unpublished data).

Histology of the frozen tissues was confirmed by inspection of H&E-stained frozen sections. Tissue specimens were microdissected to enrich tumor cells to >90% of surface area as described by Berthon et al. (48). Benign prostate tissue from the transition zone of radical prostatectomy specimens was collected from 15 of 19 patients and used as matched controls for the AD-CaP specimens. Clinical data from all patients, including age, race, serum PSA, pathologic stage, and Gleason sum grade, were acquired from a clinical database updated annually (J. L. M.). A urological pathologist (M. V. L.) blinded to other experimental data reviewed all histological diagnoses and Gleason grades.

**IHC.** Specimens were deparaffinized, rehydrated, and incubated in antigen retrieval solution (Citra (pH 6.0); BioGenex, San Ramon, CA) in a 100°C steam bath for 35 min (49). After antigen retrieval, the tissue was incubated in 2% normal goat serum for 2 min and incubated for 2 h in 5 μg/ml polyclonal rabbit antihuman HER-2 antibody (c-erbB-2 Oncoprotein; Dako Corporation, Carpinteria, CA) at 37°C. The antibody was detected with biotinylated secondary antibody (goat antirabbit IgG) for 15 min at 37°C and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 15 min at 37°C. The
immunoperoxidase antigen-antibody reaction products were visualized by incubation in diaminobenzidine for 8 min at 37°C. The tissue was counterstained with Gill’s hematoxylin for 15 s at room temperature. The specimens were stained in two batches and each included positive controls that contained three pelleted, paraffin-embedded, formalin-fixed human breast cancer cell lines with staining intensity scores of 0, 1+, 2+, and 3+ (Dako).

HER-2 protein expression was determined by a urological pathologist (M. V. I.) who evaluated membrane-staining intensity of malignant epithelial cells using a scale 0 to 3+ (Fig. 1). A score of 0 was assigned if <10% of CaP cells stained, 1+ if >10% of CaP cells had faint and incomplete membrane staining, 2+ if >10% of CaP cells had weak to moderate and complete membrane staining, and 3+ if >10% of CaP cells had strong and complete membrane staining.

**HER-2 Message Level Determinations Using qPCR**

**q-PCR Theory.** A HER-2-specific and nonextendable oligonucleotide probe was designed and labeled with a reporter fluorescent dye, 6FAM, at the 5’ end and a quencher dye, 6-carboxytetramethylrhodamine, at the 3’ end (50). The probe was hybridized to the target cDNA between the 5’ and 3’ oligonucleotides and, as amplification proceeded, the 5’ fluorophor was cleaved off the probe by 5’ nuclease activity of the polymerase. Free in solution, the 5’ fluorophor was no longer quenched by 6FAM, which increased fluorescence at 518 nm. Fluorescence intensity produced during the PCR amplifications was monitored using the 96-well thermal cycle ABI Prism 7700 (Perkin-Elmer-ABI, Foster City, CA). A real-time amplification plot was generated for each well. The number of amplification cycles was plotted on the X-axis and the log of change in fluorescence over baseline (ΔRn = fluorescence − baseline fluorescence) on the Y-axis. The instrument’s software calculated a threshold cycle number (Ct) at which each PCR amplification reached a significant threshold level. This threshold cycle is directly proportional to the number of specific HER-2 template copies present in the sample. In contrast to end point determinations, real-time quantitation establishes levels of templates long before the amplification curve flattens because of depletion of reaction components (nucleotide triphosphates and enzyme). Postamplification steps such as gel electrophoresis and staining for quantitation of amplimers are obviated. These considerations make real-time fluorescent quantitative PCR much more accurate and precise compared with previous methods of determining message levels using PCR.

**HER-2-specific mRNA Assay.** The cDNA sequence for HER-2 was obtained from GenBank using the GCG analysis programs (University of Wisconsin). HER-2-specific 5’ and 3’ oligos and an intervening fluorescent dye-labeled probe were fashioned using Primer Express (ABI/Perkin-Elmer). The probe was synthesized, labeled with 3’ 6-carboxytetramethylrhodamine, 5’ 6FAM, and high-performance liquid chromatography purified (Integrated DNA Technologies, Coralville, IA).

HER-2 sRNA was used as positive control and absolute
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standard in all assays. In brief, full-length HER-2 cDNA was subcloned into the pGEM7ZF+ vector bearing the T7 promoter. This linearized construct was used to in vitro transcribe HER-2 cRNA using the MEGAscript kit (Ambion, Austin, TX). Amplification of 2-fold serial dilutions of the HER-2 sRNA was used to construct standard curves and determine the dynamic range of the assay. Using this absolute standard, HER-2 message levels were measured accurately over the five-log range from 200 to 90 million template copies (0.1 femtograms to 2 × 10⁸ femtograms; Fig. 2A).

Inaccuracies of RNA quantitation and pipetting inefficiency can introduce well-to-well and sample-to-sample RNA loading differences. Ribogreen (Molecular Probes, Eugene, OR) fluorescence was used to quantitate starting levels of 10 ng/μl total RNA extracted from the tumor samples. A one-step RT-PCR approach was used to additionally reduce inaccuracies by avoiding synthesis of cDNA under differing reaction conditions and reducing the number of pipetting steps.

RNA Isolation

Total RNA was isolated using a guanidinium isothiocyanate protocol (RNAesy, Qiagen, Valencia, CA). Snap-frozen tumor specimens were power homogenized (Power Gen 125; Fisher Scientific, Pittsburgh, PA) before chloroform extraction. DNA contamination was resolved by treating all total RNA isolates with DNase (RNase free; Ambion). Each mRNA unknown was tested for DNA contamination by including reactions lacking the reverse transcriptase as a control.

All tissue sample RNAs were tested for HER-2 expression levels on the same day using the same master reaction mix to minimize experimental variability. Each tissue sample was tested in triplicate and the mean femtogram expression level was converted to copy number using the formula (6.02 × 10⁻⁶ copies/mol × (measured HER-2 grams/molecular weight of HER-2 message). The molecular weight of the HER-2 sRNA calculated from its nucleotide sequence was 1,732,705 g/mol.

FISH

FISH was performed using PathVysion HER-2 DNA Probe and Paraffin Pretreatment Reagent kits (Vysis, Inc., Downers Grove, IL). In brief, paraffin sections were deparaffinized, dehydrated in ethanol and air-dried. Specimens were treated sequentially with acid, chaotrope, and protease. HER-2 was labeled with SpectrumOrange, and the CEP 17 was labeled with SpectrumGreen. As an internal control for the analysis of HER-2, CEP 17 was used in a dual hybridization with HER-2 probe. After specimens were post fixed, slides were denatured at 73°C for 5 min and 10 μl of probe, and hybridization mix was placed on the slide that was cover slipped and sealed with rubber cement. Slides were hybridized overnight at 37°C and washed at room temperature just long enough to remove cover slips in 2× SSC 0.3% NP40 and then in the same solution at 70°C for 2 min. Nuclei were counterstained with 1 μg/ml DAPI in p-phenylenediamine dihydrochloride.

Hybridization signals were counted in 60 nuclei/specimen using criteria for analysis described previously (51). Analyses were performed using a fluorescence microscope (Axioskop; Zeiss, Inc.) equipped with a triple-pass filter for simultaneous detection of SpectrumGreen, SpectrumOrange, and DAPI (DAPI/Green/9-Orange; Vysis, Inc.) or a dual-pass filter (DAPI/Green or DAPI/9-Orange). Individual signals for HER-2 and CEP 17 were recorded and results reported as the HER-2 copy number divided by CEP 17 copy number; ratios ≥ 2.0 signified HER-2 amplification.

Data Analysis

Gleason sums were divided into three groups for analytical purposes: Gleason sums 5, 6, 7, and 8–10. Pathological stages were divided into three groups: stages pT2, pT3, and pN+. The Fisher’s exact test (two-tailed) was used to search for differences in the intensity of HER-2 receptor immunostaining between AD-CaP and AI-CaP from 25 Caucasian and 25 African Americans. Logistic regression models were used to compare intensity of HER-2 receptor immunostaining with age and serum PSA levels (μg/ml) for the 50 patients with AD-CaP. AI-CaP patients were not included in these analyses because HER-2 receptor immunostaining was found in only one of these specimens.

Null hypothesis was framed in the following manner to statistically test the relationship between mean HER-2 qPCR copy number between CaP and benign prostate. The mean of qPCR-CAP was greater than the mean of qPCR-BPH by at least 30% of the mean of qPCR-CAP, or the mean of qPCR-CAP was less than the mean of qPCR-BPH by at least 30% of the mean of qPCR-CAP. Our alternative scientific hypothesis was that these means were equivalent. Welch’s approximate procedure
for unknown and unequal variances was used that yields a $t$-distributed variable with $df$ degrees of freedom. The equation used to figure $t$ statistics was:

$$t = \frac{(\bar{X}_{BPH} - \bar{X}_{CAP}) \pm 0.30\bar{X}_{CAP}}{\sqrt{s^2_1/n_1 + s^2_2/n_2}}$$

where:

- $\bar{X}_{CAP}$ = the sample mean of qPCR-CaP
- $\bar{X}_{BPH}$ = the sample mean of qPCR-benign prostate
- $s_1$ = the sample variance of qPCR-CaP
- $s_2$ = the sample variance of qPCR-benign prostate
- $n_1$ = the number of qPCR-CaP measurements
- $n_2$ = the number of qPCR-benign prostate measurements

Welch’s approximation for the degrees of freedom ($df$) is:

$$df = \frac{\left(\frac{s^2_1}{n_1} + \frac{s^2_2}{n_2}\right)^2}{\frac{s^2_1}{n_1 - 1} + \frac{s^2_2}{n_2 - 1}}$$

To demonstrate equivalency of copy numbers between CaP and benign prostate, both of the null hypotheses must have been rejected. TOSTs were conducted, both of which had to be significant to demonstrate equivalency (or evidence that there is neither a 30% inferiority nor a 30% superiority in the mean difference). The critical $t$ values for this comparison (for $\alpha = 0.05$) were $-2.04$ and $2.04$. Data revealed a $t$ of $-2.13$ ($-2.13 < -2.04$) for one TOST and a $t$ of $3.81$ ($3.81 > 2.04$) for the other TOST. Thus both null hypotheses were rejected (at $\alpha = 0.05$), and the means for qPCR-CaP and qPCR-BPH were equivalent.

### RESULTS

**HER-2 Protein Expression by IHC.** Of the 50 AD-CaP specimens, 26 (52%) immunostained for HER-2 receptor (Table 1). Only 1 of 25 (4%) AI-CaP specimens immunostained for HER-2. HER-2 receptor immunostaining was greater in AD-CaP than AI-CaP ($P < 0.0002$). HER-2 receptor immunostaining was not observed in the stroma.

Among AD-CaP specimens (Table 2), there was no correlation between HER-2 receptor immunostaining and age ($P = 0.83$, data not shown), PSA ($P = 0.14$, data not shown), pathological stage ($P = 0.90$) and Gleason sums ($P = 0.13$). Finally, HER-2 receptor immunostaining did not differ significantly between Caucasian and African Americans ($P = 0.80$).

**HER-2 mRNA Levels Assayed by qPCR Are not Higher in AD-CaP Compared with Matched Benign Prostate.** AD-CaP specimens revealed a range of 13,502 to 39,850 and mean 22,080 ± 7,971 HER-2 copies/10 ng of total tumor RNA. Matched benign prostate ranged from 14,062 to 30,574 with a mean 23,957 ± 4,936 HER-2 copies/10 ng of total RNA. Ratios of HER-2 message levels from matched specimens of AD-CaP and benign prostate are depicted in Fig. 3; the highest ratio was 1.6:1, and the lowest was 0.59. Of the 15 matched sets, 7 patients revealed ratio greater than one and eight less than one.

AI-CaP specimens revealed a range from 4,505 to 22,037 and mean 15,496 ± 5,707 HER-2 copies/10 ng of total RNA.

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**Table 1** Visual scoring of HER-2 immunostaining in AD-CaP versus AI-CaP

<table>
<thead>
<tr>
<th>Race</th>
<th>No. of patients</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
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<tr>
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</tr>
<tr>
<td>African American</td>
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<td>11</td>
<td>9</td>
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**Table 2** Visual scoring of HER-2 mRNA immunostaining in AD-CaP classified by Gleason grade, pathologic stage, and race

<table>
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<th>Pathologic stage</th>
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<td>pT2</td>
<td>14</td>
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**Table 3** HER-2 mRNA copies in benign, AD-CaP, and AI-CaP specimens

<table>
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<th>Tissue Type</th>
<th>n</th>
<th>Range</th>
<th>Mean ± SD</th>
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<tr>
<td>Benign</td>
<td>15</td>
<td>14,062–30,574</td>
<td>23,957 ± 4,936</td>
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<tr>
<td>AD-CaP</td>
<td>19</td>
<td>13,502–39,856</td>
<td>22,080 ± 7,971</td>
</tr>
<tr>
<td>AI-CaP</td>
<td>14</td>
<td>4,505–22,037</td>
<td>15,496 ± 5,707</td>
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</table>

*$P < 0.01$, benign versus AD-CaP.

*$P < 0.05$, AD-CaP versus AI-CaP.
(Table 3). The mean values were lower ($P < 0.05$) for AD-CaP and benign prostate. The specimen from patient 60 was the only one that demonstrated HER-2 protein expression by IHC, and this specimen demonstrated the highest level of mRNA expression (22,037 copies/20 ng of total RNA).

**FISH.** HER-2 amplification was not detected in any of 39 androgen-dependent or androgen-independent CaP specimens tested (Fig. 4 and Table 4). There were no differences in the HER-2: CEP 17 oncogene-to-centromere 17 ratios by androgen status (1.43 ± 0.22 AD-CaP and 1.34 ± 0.21 AI-CaP) or race (1.44 Caucasian and 1.35 African Americans).

**DISCUSSION**

IHC Quantitation of HER-2 Protein Expression Levels Remains Elusive. The paradigm that overexpression of cell surface receptors can lead to malignant transformation of cells has been fruitful. IHC has been invaluable for qualitative study of expression of gene products in tumor specimens; however,
quantification remains problematic even for extensively developed targets such as HER-2 (52, 53). The published IHC data on HER-2 expression levels for CaP are emblematic of the difficulties encountered in attempting IHC quantitation (Table 5; Refs. 31–34, 37–40, 41, 43, 45–47, 54–57). In the 18 studies cited, 9 different anti-HER-2 antibodies were used, and the definition of overexpression was similarly disparate. The reported expression levels range from 0 to 100% of samples studied. Specifically, Kuhn et al. (33) and Zhou et al. (34) found no HER-2 receptor immunostaining in 11 (9 and 2 specimens, respectively) paraffin-embedded BPH specimens, whereas Ware et al. (35) reported that all 11 fresh-frozen benign prostate specimens immunostained for HER-2 receptor. McCann et al. (36) found no HER-2 receptor immunostaining in 23 paraffin-embedded CaP specimens, whereas Gu et al. (32) report that HER-2 receptor was found in all of 39 paraffin-embedded tumors.

We used immunohistostaining methods now considered standard for HER-2 detection in breast cancer. We optimized IHC parameters, including tissue acquisition, storage, and antigen retrieval from archived tumors (49). We used the Food and Drug Administration-approved and -validated anti-HER-2 antibody from Dako and titrated its use in AD-CaP, AI-CaP, and appropriate control slides (58). We found that 19% (9 of 50) of the AD-CaP sample (IHC – Refs. 31, 34, 37–39, 57). In the 18 studies cited, 9 different anti-HER-2 antibodies were used, and the definition of overexpression was similarly disparate. The reported expression levels range from 0 to 100% of samples studied. Specifically, Kuhn et al. (33) and Zhou et al. (34) found no HER-2 receptor immunostaining in 11 (9 and 2 specimens, respectively) paraffin-embedded BPH specimens, whereas Ware et al. (35) reported that all 11 fresh-frozen benign prostate specimens immunostained for HER-2 receptor. McCann et al. (36) found no HER-2 receptor immunostaining in 23 paraffin-embedded CaP specimens, whereas Gu et al. (32) report that HER-2 receptor was found in all of 39 paraffin-embedded tumors.

As would be expected, widely disparate results are also reported regarding the correlation between HER-2 expression levels and CaP prognostic factors such as tumor grade and stage. Mellon et al. (37) and Gu et al. (32) found no significant correlation between stage or grade and HER-2 receptor immunostaining. Sadasivan et al. (31) found that HER-2 receptor immunostaining correlated with tumor grade and stage. In a more recent publication, Signoretti et al. (46) reported that HER-2 protein expression was statistically higher in tumors that had been treated with androgen deprivation therapy before resection. They also reported that the proportion of HER-2-positive tumors increased when comparing tumors resected before androgen deprivation therapy compared with those resected after androgen deprivation therapy. Although half of the AD-CaP specimens expressed HER-2 receptor, we found no relationship between the intensity of HER-2 receptor immunostaining and age, race, PSA levels, pathologic stage, or Gleason sum.

**HER-2 is Rarely Amplified in CaP.** One frequent mechanistic and empirical explanation for HER-2 overexpression is amplification of the gene. In breast cancer specimens, HER-2 amplification measured by Southern blot and, more recently, FISH strongly correlated with protein expression levels measured by IHC (51, 59). Although consensus about HER-2 IHC data remains elusive, studies of HER-2 amplification leave little doubt that amplification of this gene in CaP is a rare event (33, 34, 42, 45, 47, 60, 61). Using the well validated FISH assay (Pathysion) developed by Vysis, Inc., in collaboration with Lynn Dressler (Cancer and Leukemia Group B study 8541) (51), HER-2 amplification was not detected in any of our AD or AI CaP specimens.

Ross et al. (38) studied 113 archival CaP specimens, of which 41% had five or more copies of HER-2 in at least 20 cells of 100 studied. This series is criticized in that their FISH assay did not include a second stain to normalize for chromosomal or centromeric counts. Furthermore, only 29% of the same samples overexpressed HER-2 by IHC, and they were unable to statistically correlate amplification with overexpression. A more recent publication by the same group reported amplification in only 2 of 66 cases. Liu et al. (41) reported 53% low copy number (three or more signals/nucleus) HER-2 amplifications in CaP. These investigators do not state what percentage of the cells counted had to have three or more signals to be considered amplified, and only one of their patients had membrane staining for HER-2 protein. Similarly, Skacel et al. (44) reported low level amplification (three to five signals/nucleus corrected for chromosome 17 aneusomy) in 26% of specimens, but it is unclear what proportion of the 100 cells counted had to be abnormal to define amplification. Oxley et al. (42) studied 117 CaP specimens with HER-2 and chromosome-17 centromeric probes and enzymatic detection methods. HER-2 amplification was defined as five or more signals in each nucleus in >20% of malignant cells counted and normalized for chromosome 17 polysomy. HER-2 amplification was detected in only two (1.75%) tumors using these criteria.

**HER-2 mRNA Expression Levels Correlate with IHC and FISH Findings.** Careful tissue banking, guaumin-based total RNA extraction, and quantitation of purified RNA at the 10 ng/μl levels using ribo-green fluorescence provided high-quality starting substrate for message level determinations. Adoption of single tube RT-PCR and running all assays from one master reagent mix on the same day obviated introduction of differences in reaction conditions among samples assayed. Use of in vitro transcribed HER-2 sRNA in 3-fold dilutions as a standard titration curve with each experiment allowed absolute quantitation of message and provided an excellent measure of the sensitivity and dynamic range of the assay (Fig. 2B). HER-2 mRNA template copies [350 to 70 million (1.0–2 × 10⁵ femtograms)] were measured reproducibly from 10 ng of total RNA extracted from tissue samples.

mRNA data were consistent with FISH and IHC findings; HER-2 expression levels were not increased in AD-CaP compared with AI-CaP. HER-2 mRNA levels in AI-CaP were lower than AD-CaP (P < 0.05), which agreed with the IHC findings that only one AI-CaP expressed HER-2 versus 19% (HER-2 2+ or 3+) of AI-CaP. Furthermore, the highest HER-2 expressing AI-CaP sample (IHC = 1+) also exhibited the highest mRNA expression levels of HER-2 in the 10 ng/μl range.
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<th>Type</th>
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<td>0-3%</td>
<td>3 or more</td>
<td>53% (&gt;3 spots)</td>
</tr>
<tr>
<td>Shi 2001</td>
<td>AD-CaP 61</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Dako (1:600) (hercept</td>
<td>&gt;50% cells with stamina stronger than normal cells</td>
<td>29% of AD-CaP</td>
<td>NA</td>
</tr>
<tr>
<td>Skacel 2001</td>
<td>AD-CaP 39</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>FISH</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3-5 signals/nucleus</td>
</tr>
<tr>
<td>Osman 2001</td>
<td>AD-CaP 83</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Dako 485</td>
<td>Weak to moderate membrane staining in &gt;10% of cells (2+)</td>
<td>38%</td>
<td>NA</td>
</tr>
<tr>
<td>Reese 2001</td>
<td>AD-CaP 39</td>
<td>TURP Biopsy</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>TAB250 1:200 monoclonal</td>
<td>10% (4 cases with 2+ or 3+ staining)</td>
<td>HER-2/centromere 17 ratio ≥ 1.5</td>
<td>1 specimen</td>
</tr>
<tr>
<td>Signoretti 2001</td>
<td>AD-CaP 101</td>
<td>RP 69</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Dako 485 (1:200)</td>
<td>Immunostaining &gt; adjacent basal cells</td>
<td>25% of AD-CaP</td>
<td>0%</td>
</tr>
<tr>
<td>Haussler 1999</td>
<td>Adenosis 48</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>9G6 (1:600) monoclonal</td>
<td>0% of CaP</td>
<td>0% of PIN</td>
<td>NA</td>
</tr>
<tr>
<td>Morote 1999</td>
<td>AD-CaP 38</td>
<td>Biopsy</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>CB-11 (1:40) monoclonal</td>
<td>Positive &gt;1% of cells stained</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Myldo 1998</td>
<td>AD-CaP 14</td>
<td>RP 8</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>9G6 (1:200) monoclonal</td>
<td>3 of 17 revealed &gt;20% of cells stained</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ross 1997</td>
<td>AD-CaP 113</td>
<td>TURP 9</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Dako 485 (1:40)</td>
<td>Average combined membranous and cytoplasmic stain ≥3</td>
<td>29% (intense cytoplasmic or diffuse membranous)</td>
<td>5 or more signals in &gt;20% of nuclei</td>
</tr>
<tr>
<td>Kuhn 1993</td>
<td>AD-CaP 53</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>pAb-1 (1:20) monoclonal</td>
<td>Definite positive membranous staining Grade 2 focal staining (1 = weak to moderate, 2 = strong)</td>
<td>34%</td>
<td>NA</td>
</tr>
<tr>
<td>Mellon 1992</td>
<td>AD-CaP 29</td>
<td>TURP</td>
<td>Frozen</td>
<td>IHC</td>
<td>NCL-CB11 (1:40) (Novocastra)</td>
<td>21% of CaP</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Zhou 1992</td>
<td>AD-CaP 16</td>
<td>NS</td>
<td>Frozen</td>
<td>IHC</td>
<td>Ab-3 (5 ug/ml)</td>
<td>80% by IHC</td>
<td>Southern blots 0% (0 of 10)</td>
<td>NA</td>
</tr>
<tr>
<td>Gu 1996</td>
<td>AD-CaP 39</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Ab-3 (1 ug/ml)</td>
<td>100% of CaP were 1-3 20% of BPH</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Visakorpi 1992</td>
<td>AD-CaP 147</td>
<td>Biopsy 101</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Mab-1</td>
<td>NS</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Veltri 1994</td>
<td>AD-CaP 124</td>
<td>RP</td>
<td>Formalin-paraffin</td>
<td>IHC</td>
<td>Ab-3</td>
<td>Focal, diffuse, or none</td>
<td>78%</td>
<td>NA</td>
</tr>
<tr>
<td>Sandavisan 1993</td>
<td>AD-CaP 25</td>
<td>RP</td>
<td>Frozen</td>
<td>IHC</td>
<td>TA-1 (0.5 ug/ml) monocular</td>
<td>36% had 3+</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*ABC, avidin-biotin peroxidase; IAP, immunoalkaline phosphatase; PIN, prostatic epithelial neoplasia; TURP, Transurethral resection of prostate; RP, radical prostatectomy; Mets, metastases; NA, not applicable; NS, not specified.*
HER-2 copy number of the set. Of the matched sets where AD-CaP had copy numbers greater than benign prostate, none had ratios >2X, which contrasts with results reported for breast cancer where HER-2 overexpression usually means 4X background or greater (unpublished data).

In vitro model systems suggest that HER-2 overexpression may play a role in progression of CaP to androgen independence. However, hypotheses generated by model systems need to be validated in primary tumor specimens. Our three independent sets of data are consistent and do not support a role for stable HER-2 overexpression in either prostatic carcinogenesis or CaP progression to androgen independence. Solid tumors are comprised of heterogeneous groups of cells that are unlikely to become androgen independent in concert. A more probable course is that a subset of the androgen-dependent tumor cells progress to androgen independence and eventually outgrow the androgen-dependent subpopulation; this is the reason AI-CaP was examined after a long period of androgen deprivation therapy. Some of the in vivo animal models suggest that transient HER-2 induction is required to overcome growth arrest or apoptotic signals as a result of androgen deprivation therapy. In this scenario, extracting RNA from the total tumor mass would dilute the increased HER-2 message levels experienced by a few tumor cells. An experimental solution to this issue is to extract RNA from areas of a tumor expressing high levels of HER-2 (as detected by IHC) using laser capture microdissection of OCT-embedded snap-frozen tumor. Although total RNA has been obtained from OCT frozen tumor samples via laser capture microdissection, the quantity has been insufficient for quantitation of HER-2.

In summary, HER-2 receptor at 2+ or 3+ IHC levels was detected in 19% of AD-CaP, but its expression was unrelated to age, race, serum PSA, and pathologic stage and grade. AI-CaP rarely immunostained for HER-2 receptor. The lack of HER-2 overexpression in AI-CaP found by IHC was confirmed by careful quantitative measurement of HER-2 mRNA and gene amplification.

The past two decades have seen the concurrent evolution of three currents: discovery of the HER1–4 receptor-ligand family; incremental understanding of the complexity of interactions among these and evolution of monoclonal antibody; and qPCR and FISH technologies. The easy availability of paraffin-embedded tumors coupled with the earlier development of IHC methods compared with FISH and Q-PCR explains, in part, the focus on HER-2 IHC studies. Given that mRNA for all family members (HER1–4) is detected in benign prostate, AD-CaP and AI-CaP, correlations with outcome, biological transition to androgen independence, or response to therapy may require study of all family members in concert. We have outlined carefully the difficulties in quantitating IHC preparations and believe strongly that synchronous evaluation of gene copy number and message level facilitate a more critical view of each individual data set. Given the biological importance of the HER1–4 receptors that are often activated by autocrine or paracrine ligands in tumors, novel therapies that target family members may indeed prove effective. However, it is not yet evident that overexpression is a requirement for efficacy; it is more likely that levels of expression will modulate the efficacy of these exciting novel therapeutic agents. Indeed Agus et al. (62) have shown that antibody 2c4 that inhibits ligand binding by HER-2 is effective in suppressing growth of xenografted tumors that do not overexpress HER-2.

REFERENCES


HER-2 Expression in Prostate Cancer


Human Epidermal Receptor-2 Expression in Prostate Cancer

Benjamin F. Calvo, Aaron M. Levine, Mavie Marcos, et al.


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