Quantitative Analysis of Breast Cancer Tissue Microarrays Shows That Both High and Normal Levels of HER2 Expression Are Associated with Poor Outcome

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Abstract

Using a tissue microarray cohort of 300 breast cancers and 84 samples of normal breast epithelium, we analyzed HER2/neu expression and compared traditional clinical (manual) scoring with a recently developed system for the quantitative measurement of immunohistochemical stains (AQUA). As expected, both methods identified a population (10–15%) of high-HER2-expressing tumors with poor 30-year disease-related survival. Using AQUA analysis, we found that normal epithelium expresses a low but detectable level of HER2 and that 17.5% of tumors exhibit similar low-level HER2 expression. This low group was not definable by manual scoring. Surprisingly, HER2-normal tumors were as aggressive as HER2-overexpressing tumors. Our studies suggest that in situ quantitative measurement of HER2 stratifies breast tumors into three expression levels: normal, intermediate, and high, where both normal and high levels are associated with a worse outcome.

Introduction

HER2 (neu or erb-B2), a member of the epidermal growth factor family, is genetically amplified and overexpressed in aggressive breast cancers. High levels of HER2 are associated with poor prognosis, particularly in node-positive breast carcinoma patients. Recently, a targeted therapeutic against HER2 has been developed. Trastuzumab (Herceptin) is a humanized monoclonal antibody directed against the extracellular domain of HER2. Treatment of patients with metastatic breast carcinoma with Herceptin has shown therapeutic benefit, especially when combined with conventional chemotherapy agents. The association between HER2 expression and Herceptin response has stimulated renewed interest in accurately assessing HER2 amplification and overexpression. Toward this goal, we have developed a system for compartmentalized, automated quantitative analysis of histological sections (AQUA; Ref. 1). As with an ELISA, AQUA provides highly reproducible analysis of target signal expression with use of a continuous, rather than nominal, scale. Unlike an ELISA, spatial information, including tissue and subcellular localization, is preserved. Using a tissue microarray composed of archival breast cancer specimens and normal epithelia, we found a bimodal distribution of HER2, where tumors expressing both high and normal HER2 levels exhibited poor 30-year disease-specific survival.

Materials and Methods

Tissue Microarray Design. Paraffin-embedded, formalin-fixed specimens from 300 cases of node-positive invasive breast carcinoma were identified from the archives of the Yale University Department of Pathology as available from 1962 to 1977, with a mean follow-up time of 9.6 years. No patients received Herceptin during the study period. Complete treatment information was unavailable for the entire cohort; however, most patients were treated with local radiation and ~15% were treated with chemotherapy consisting primarily of Adriamycin, cytoxan, and 5-fluorouracil. Approximately 27% subsequently received tamoxifen (post-1978). Seven patients had biopsy-proven stage IV disease at the time of diagnosis.

In constructing the microarrays, we identified areas of invasive carcinoma, away from in situ lesions and normal epithelium, and took two 0.6-mm cores. We cut 5-µm-thick sections of the microarrays and processed them as described previously (2, 3). We previously demonstrated with HER2 that two cores replicated the results of an entire slide in >95% of cases (4). An additional microarray consisting of 84 samples of normal epithelium was also constructed from samples of normal ducts and lobules taken from breast cancer patients. Samples were taken away from areas of tumor and assessed histologically to ensure that they were unaffected by atypical hyperplasia or carcinoma in situ.

Immunohistochemistry. Tissue microarray slides were stained as described (1). In brief, for both manual and automated analysis, slides were incubated for 1 h at room temperature with polyclonal anti-HER2 (1:200; DAKO Corp., Carpinteria, CA) diluted in Tris-buffered saline containing BSA. We cut 5-µm-thick sections of the microarrays and processed them as described (1). In brief, for both manual and automated analysis, slides were visualized with diamino benzenidine (Dako Corp.,), followed by ammonium hydroxide-acidified hematoxylin. For automated analysis, tumor cells were distinguished by use of a fluorescently tagged anticytokeratin antibody cocktail (AE1/AE3; Dako Corp.). We added 4',6-diamidino-2-phenylindole to visualize nuclei, and HER2 was visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence.

Automated Image Acquisition and Analysis. Automated image acquisition and analysis using AQUA has been described previously (1). In brief, monochromatic, high-resolution (1024 × 1024 pixel; 0.5-µm) images were obtained of each histoslot. We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal. Coalescence of cytokeratin at the cell surface helped localize the cell membranes, and 4',6-diamidino-2-phenylindole was used to identify nuclei. The HER2 signal from the membrane area of tumor cells was scored on a scale of 0–255 and expressed as signal intensity divided by the membrane area.

FISH. FISH4 analysis was performed with the PathVysion HER2 DNA Probe Kit (Vysis, Downs Grove, IL), using two directly labeled fluorescent DNA probes complementary to the HER2/neu gene locus (LSI HER2/neu SpectrumRed) and to chromosome 17 pericentromeric α satellite DNA (CEP17 SpectrumGreen), according to standard protocols. HER2/neu gene amplification was quantified by comparing the ratio of LSI HER2/neu to CEP17 probe signals in accordance with the PathVysion HER2 DNA Probe Kit criteria. We examined 60 nonoverlapping tumor cell nuclei in each histo-
HER2 EXPRESSION ASSOCIATED WITH BREAST CANCER OUTCOME

Results and Discussion

Validation of Microarray Cohort. To validate our tissue microarray cohort of 300 node-positive breast cancers, we assessed several traditional histopathological markers of malignancy. Using univariate analysis of long-term disease-related survival, we found that large tumor size, high nuclear grade, low estrogen receptor expression, and high number of involved lymph nodes were all significant predictors of poor outcome (Table 1). We next assessed the prognostic power of HER2 immunohistochemistry, using standard brown staining, visual examination by a pathologist, and scoring on a four-point scale (0 to 3+). Manual analysis showed a typical pattern of HER2 expression with 15% of tumors overexpressing the antigen (2+ and 3+; Fig. 1B). As expected, high-level (3+) tumors showed a significantly worse outcome with a relative risk of 2.25 (P = 0.0007; Table 1). Analysis of HER2 gene amplification by FISH was not predictive in our study, but this was most likely attributable to the relatively small number of cases that, for technical reasons, were scorable (125 of 300; Table 1). However, both automated and manual analyses of HER2 protein levels were highly correlated with HER2 gene amplification (P < 0.0001). The percentage of HER2-amplified cases in each manual category were 4.0% (0), 13.7% (1), 71.4% (2), and 75.0% (3+), and in each AQUA category were 9.5% (normal), 13.7% (intermediate), and 77.8% (high).

HER2 Expression on Normal Epithelium. We then assessed the level of HER2 expression on normal breast epithelium with use of automated analysis on a microarray. This epithelium was derived from normal ducts and/or lobules isolated from uninvolved breast tissue taken from 84 breast cancer patients. Consistent with previous studies using biochemical assays, our results demonstrated a low but detectable level of HER2 in normal epithelium, which was tightly grouped into a single peak with a mean of 5 and a SD of 1.5 (AQUA score: Fig. 1A; Ref. 6).

Automated Analysis of HER2 Expression in Breast Cancer. In contrast to the tightly grouped peak in normal epithelium, HER2 expression in breast tumors was broadly distributed (Fig. 1C). Expression levels of HER2 in tumors exhibited a mode similar to that of normal epithelium, but with significant skew toward higher-level expression. Examination of the histogram suggested that there were three naturally occurring populations based on HER2 expression: normal, intermediate, and high (Fig. 1C). A discernible break in the histogram at AQUA score 25 divided HER2-high from the remaining tumors. The remaining tumors could then be subdivided into HER2-low and HER2-intermediate groups depending on whether their expression levels were greater than the mean HER2 expression on normal epithelium + 1 SD (AQUA score <6.5; Fig. 1, A and C). On the basis of these divisions, 17.5% of the tumors were designated HER2 normal, 71.3% were HER2 intermediate, and 11.2% were HER2 high.

Comparison of Manual and Automated Techniques. We then compared HER2 expression as gauged by automated and manual techniques (Fig. 1, panels C and B, respectively). In contrast to AQUA scores, which were continuously scored on a scale of 0–255, manual scoring of HER2 expression was performed on a nominal four-point scale (0 to 3+). Despite this difference, regression analysis demonstrated good correlation between the two methods (r = 0.704). However, there was a significant degree of overlap in the automated scores of cases from adjacent manually determined groups (Fig. 1D). Whereas there was a clear division between the histograms of tumors scoring 0/1+ and 2+/3+, the distinction between tumors scoring 0 and 1+ was indistinct. This result shows the difficulty in manually translating a biological (continuous) marker into a nominal four-point scale. Even for the trained eye of a pathologist, accurate distinction between nominal categories (e.g., 2+ versus 3+) is difficult and often arbitrary. Indeed, recent studies have demonstrated a significant lack of reproducibility in the clinical determination of HER2 levels attributable in part to this difficulty (7–9).

Examination of manual and automated techniques revealed that both were equally able to define a population of tumors expressing high levels of HER2 with poor outcome (relative risk, 2.25 and 2.18; P = 0.0007 and 0.0013, respectively; Table 1). However, unlike manual analysis, automated analysis revealed that tumors expressing normal levels of HER2 also showed a significantly worse outcome (relative risk, 1.71; P = 0.0091; Table 1). Given the amount of overlap in the 0 and 1+ categories from manual scoring (Fig. 1D), it is not surprising that manual assessment of stained slides has not previously identified the HER2-normal population.

Defining the Subpopulation of HER2-normal Tumors. To determine whether HER2 expression correlated with known prognostic markers in our cohort, we assessed possible associations between HER2 and hormone receptor status, tumor size, and nuclear grade. High-level HER2 expression was correlated with high nuclear grade and inversely correlated with estrogen receptor status (Table 2). The HER2-normal population showed no significant correlation with nodal involvement, tumor size, or estrogen receptor, but did show an association with high nuclear grade (P = 0.0494; Table 2). Few of the HER2-normal tumors exhibited gene amplification (2 of 21 examined), ruling out the possibility that in tumors expressing

<table>
<thead>
<tr>
<th>Marker</th>
<th>n</th>
<th>P</th>
<th>Relative risk</th>
<th>95% confidence interval</th>
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<tr>
<td>HER2 manual score</td>
<td>0.0071</td>
<td>153</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>1+</td>
<td>50</td>
<td>0.0383</td>
<td>1.02</td>
<td>0.68–1.52</td>
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<tr>
<td>2+</td>
<td>13</td>
<td>0.9763</td>
<td>1.01</td>
<td>0.49–2.08</td>
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<tr>
<td>3+</td>
<td>28</td>
<td>0.0007</td>
<td>2.25</td>
<td>1.41–3.58</td>
</tr>
<tr>
<td>HER2 AQUA score</td>
<td>0.0009</td>
<td>46</td>
<td>0.0091</td>
<td>1.71</td>
</tr>
<tr>
<td>Normal</td>
<td>Intermediate</td>
<td>0.0013</td>
<td>2.18</td>
<td>1.35–3.51</td>
</tr>
<tr>
<td>High</td>
<td>HER2 amplification (FISH)</td>
<td>0.0279</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Nodal involvement</td>
<td>1–3</td>
<td>68</td>
<td>0.6708</td>
<td>1.08</td>
</tr>
<tr>
<td>4–9</td>
<td>141</td>
<td>0.0086</td>
<td>1.62</td>
<td>1.13–2.33</td>
</tr>
<tr>
<td>≥10</td>
<td>Tumor size (cm)</td>
<td>0.0007</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>&lt;2</td>
<td>53</td>
<td>0.1255</td>
<td>1.33</td>
<td>0.92–1.93</td>
</tr>
<tr>
<td>≥2</td>
<td>102</td>
<td>0.0001</td>
<td>2.09</td>
<td>1.43–3.07</td>
</tr>
<tr>
<td>Nuclear grade</td>
<td>High</td>
<td>95</td>
<td>0.0040</td>
<td>1.55</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>Negative</td>
<td>104</td>
<td>0.0262</td>
<td>1.41</td>
</tr>
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</table>

**Table 1** Univariate analysis of 30-year disease-related survival.

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*HER2 EXPRESSION ASSOCIATED WITH BREAST CANCER OUTCOME*

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normal levels of HER2, the HER2 gene is amplified but the HER2 protein is not detected.

**Multivariate Analysis of HER2-normal and -high Populations.**

Finally, we determined whether normal or high expression of HER2 by tumors was an independent predictor of long-term disease-related survival. Combined multivariate analysis of HER2 with the traditional histopathological markers, nodal involvement, tumor size, nuclear grade, and estrogen receptor, demonstrated that both normal- and high-level HER2 expression were independently predictive of patient outcome (Table 3).

Our data suggest that HER2 divides cases of node-positive breast carcinoma into three categories: normal, intermediate, and high expressers. Tumors expressing either normal or high HER2 levels do poorly in long-term follow-up. Of particular note are three previous studies that have looked at HER2 expression levels using "gold standard" biochemical techniques (Western blots and ELISAs; Refs. 6, 10–13). Two of these studies suggested a bimodal distribution for HER2, with both low and high levels correlating with known markers of tumor aggression (10–12), but a third found no such distribution (13). Because such techniques require fresh tissue for analysis, they were unable to assess long-term follow-up on a large cohort of patients. The AQUA-based analysis provides quantitative information from tissue microarrays constructed from archival tissues; we thus were able to examine a large cohort of patients with known long-term disease-related survival. Our data show that normal HER2 expression is an independent prognostic indicator of poor outcome and demonstrate that, unlike manual immunohistochemical analysis, automated analysis can identify a patient population that is otherwise detectable only by established biochemical assays.

HER2 overexpression can induce an aggressive phenotype via the activation of downstream regulators (e.g., phosphoinositol 3-kinase, Erk/MAP kinase, and Ras; Refs. 14–16). How normal levels of HER2 could be associated with a similar aggressive phenotype is unknown at present. We speculate that these tumors might overexpress another growth factor receptor that promotes tumor aggression via a ligand-dependent or -independent mechanism. It is possible that expression

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**Table 2.** Distribution of prognostic markers by HER2 level based on $\chi^2$ analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>All cases</th>
<th>Normal (%)</th>
<th>Intermediate (%)</th>
<th>High (%)</th>
<th>$P$ (\chi^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Intermediate</td>
<td>High</td>
<td>Normal vs. intermediate</td>
</tr>
<tr>
<td>Nodes positive</td>
<td>268</td>
<td>54</td>
<td>60</td>
<td>34</td>
<td>0.8171</td>
</tr>
<tr>
<td>1–3</td>
<td>145</td>
<td>26</td>
<td>23</td>
<td>32</td>
<td>0.3033</td>
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<tr>
<td>4–9</td>
<td>70</td>
<td>20</td>
<td>17</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>53</td>
<td>20</td>
<td>35</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>238</td>
<td>43</td>
<td>35</td>
<td>32</td>
<td></td>
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<tr>
<td>&lt;2</td>
<td>102</td>
<td>34</td>
<td>44</td>
<td>20</td>
<td></td>
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<tr>
<td>2–5</td>
<td>81</td>
<td>23</td>
<td>21</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>55</td>
<td>23</td>
<td>17</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Nuclear grade</td>
<td>269</td>
<td>36</td>
<td>30</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>97</td>
<td>36</td>
<td>34</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>263</td>
<td>40</td>
<td>39</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>105</td>
<td>40</td>
<td>39</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

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of such alternate growth factor receptors in some tumors results in the down-regulation of HER2 expression via a feedback mechanism, producing aggressive tumors bearing a HER2-normal phenotype. Another possible explanation for the poor prognosis of HER2-normal tumors is that high levels of coreceptor ligand-independent activation of HER2 might result in the internalization and degradation of the receptor, producing apparent low-level HER2 expression. Finally, HER2-normal breast cancers may represent a population of aggressive poorly differentiated neoplasms that have developed HER2- and growth factor-independent mechanisms for their growth. The association between normal HER2 expression levels and high nuclear grade supports this idea. Recent data from the Brown and Botstein group supports this idea. They showed five unique breast cancer subtypes based on expression patterns of breast carcinoma genes that distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA, 98: 1069–1074, 2001.


References


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