

Quantitative Analysis of Breast Cancer Tissue Microarrays Shows That Both High and Normal Levels of HER2 Expression Are Associated with Poor Outcome¹

Robert L. Camp,² Marisa Dolled-Filhart, Bonnie L. King, and David L. Rimm

Departments of Pathology [R. L. C., D. L. R.], Genetics [M. D-F.], and Therapeutic Radiology [B. L. K.], Yale University, School of Medicine, New Haven, Connecticut 06520

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 chemotherapeutic 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- μ m-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. Previous analysis of titrations of the HER2 antibody demonstrated that higher dilutions of anti-HER2 antibody (1:1000~1:8000) more accurately define the HER2-high from the HER2-intermediate populations, whereas lower dilutions (1:50~1:500) distinguish the HER2-normal from HER2-intermediate populations.³ In this study we used a concentration (1:200) that sufficiently distinguished all three populations. Goat antirabbit antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp.) was used as a secondary reagent. For manual analysis, slides were visualized with diaminobenzidine (DAKO Corp.), followed by ammonium hydroxide-acidified hematoxylin. For automated analysis, tumor cells were identified 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- μ m) images were obtained of each histospot. 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, Downers 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-

Received 11/6/02; accepted 2/14/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from the Patrick and Catherine Weldon Donaghue Foundation for Medical Research, by grants from the NIH [K0-8 ES11571, NIEHS (to R. L. C.), and RO-1 GM57604 NCI (to D. L. R.)], the Greenwich Breast Cancer Alliance, and by United States Army DAMD Grant 01-000436.

² To whom requests for reprints should be addressed, at Department of Pathology, Yale University, School of Medicine, New Haven, CT 06520-8023.

³ R. L. Camp, M. Dolled-Filhart, D. L. Rimm, unpublished observations.

⁴ The abbreviation used is: FISH, fluorescence *in situ* hybridization.

spot to determine the average number of HER2/*neu* and chromosome 17 copies/cell for each tissue specimen. The ratio of these averages was used to determine the presence of HER2/*neu* gene amplification. Specimens with a HER2/*neu*:chromosome 17 ratio >2 were scored as positive for HER2/*neu* gene amplification.

Data Analysis. Manual scoring of HER2 expression was assessed by a pathologist (R. L. C.) using a nominal four-point scale (0 to 3+). Histospots containing <10% tumor, as assessed either subjectively (manual) or by mask area (automated), were excluded from further analysis. Previous studies have demonstrated that the staining from a single histospot provides a sufficiently representative sample for analysis (4, 5). Correlations with other prognostic markers were determined by χ^2 analysis. Overall survival analysis was assessed by Kaplan-Meier analysis with the Mantel-Cox log-rank score for determining statistical significance. Relative risk was assessed by the univariate and multivariate Cox proportional hazards model. Analyses were performed with Statview 5.0.1 (SAS Institute, Cary, NC). Patients were deemed "uncensored" if they died of breast cancer within 30 years of their initial date of diagnosis.

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 1 Univariate analysis of 30-year disease-related survival

Marker	n	P	Relative risk	95% confidence interval
HER2 manual score		0.0071		
0	153		1.00	
1+	50	0.9383	1.02	0.68–1.52
2+	13	0.9763	1.01	0.49–2.08
3+	28	0.0007	2.25	1.41–3.58
HER2 AQUA score		0.0009		
Normal	46	0.0091	1.71	1.14–2.56
Intermediate	188		1.00	
High	30	0.0013	2.18	1.35–3.51
HER2 amplification (FISH)	22	0.8121	1.07	0.60–1.90
Nodal involvement		0.0279		
1–3	68		1.00	
4–9	54	0.6708	1.08	0.75–1.55
≥10	141	0.0086	1.62	1.13–2.33
Tumor size (cm)		0.0007		
<2	80		1.00	
2–5	53	0.1255	1.33	0.92–1.93
>5	102	0.0001	2.09	1.43–3.07
Nuclear grade				
High	95	0.0040	1.55	1.15–2.08
Estrogen receptor				
Negative	104	0.0262	1.41	1.041–1.906

Table 3 Multivariate analysis of 30-year disease-related survival

Marker	n	P	Relative risk	95% confidence interval
HER2		0.0097		
Normal	42	0.0191	1.68	1.09–2.59
Intermediate	162		1.00	
High	25	0.0136	1.96	1.15–3.36
Nodal involvement		0.1058		
1–3	60		1.00	
4–9	48	0.5915	1.12	0.73–1.72
≥10	121	0.0353	1.61	1.03–2.53
Tumor size (cm)		<0.0001		
<2	78		1.00	
2–5	52	0.2220	1.31	0.85–2.01
>5	99	<0.0001	2.59	1.67–4.02
Nuclear grade				
High	87	0.2158	1.26	0.87–1.82
Estrogen receptor				
Negative	89	0.0032	1.75	1.21–2.54

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 also support this finding. They showed five unique breast cancer classes by cDNA array clustering experiments, two of which had very poor outcomes. One of these groups was HER2 positive, but the other showed no evidence of HER2 overexpression (17).

From a clinical perspective, response to Herceptin has largely been seen in HER2 high expressers or HER2-amplified cases. This may be attributable to the fact that 2+ or 3+ levels of expression were required for entry into most clinical trials (18–20). The response of 0 or 1+ tumors to paclitaxel with and without Herceptin is being studied in a large randomized trial (CALGB 9840; Ref. 21). Although patients with HER2-normal tumors are unlikely to respond to Herceptin, they may benefit from more aggressive traditional chemotherapy. The ability to accurately distinguish between HER2-normal and HER2-intermediate tumors by automated analysis not only has prognostic value but may also help in the development and evaluation of new therapeutics targeted to treat this subpopulation.

Acknowledgments

We thank Michael DiGiovanna and D. Craig Allred for critical review of this manuscript. We also thank Nicole Parisot, Thomas D'Aquila, Mary Helie, Lori Charette, and Diana Fischer for assistance in this effort.

References

- Camp, R. L., Chung, G. G., and Rimm, D. L. Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat. Med.*, 8: 1323–1328, 2002.

- Rimm, D. L., Camp, R. L., Charette, L. A., Olsen, D. A., and Provost, E. Amplification of tissue by construction of tissue microarrays. *Exp. Mol. Pathol.*, 70: 255–264, 2001.
- Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., and Kallioniemi, O. P. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat. Med.*, 4: 844–847, 1998.
- Camp, R. L., Charette, L. A., and Rimm, D. L. Validation of tissue microarray technology in breast carcinoma. *Lab. Invest.*, 80: 1943–1949, 2000.
- Torhorst, J., Bucher, C., Kononen, J., Haas, P., Zuber, M., Kochli, O. R., Mross, F., Dieterich, H., Moch, H., Mihatsch, M., Kallioniemi, O. P., and Sauter, G. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. *Am. J. Pathol.*, 159: 2249–2256, 2001.
- Dittadi, R., Donisi, P. M., Brazzale, A., Marconato, R., Spina, M., and Gion, M. Immunoenzymatic assay of erbB2 protein in cancer and non-malignant breast tissue. Relationships with clinical and biochemical parameters. *Anticancer Res.*, 12: 2005–2010, 1992.
- Paik, S., Bryant, J., Tan-Chiu, E., Romond, E., Hiller, W., Park, K., Brown, A., Yothers, G., Anderson, S., Smith, R., Wickerham, D. L., and Wolmark, N. Real-world performance of HER2 testing—National Surgical Adjuvant Breast and Bowel Project experience. *J. Natl. Cancer Inst. (Bethesda)*, 94: 852–854, 2002.
- Roche, P. C., Suman, V. J., Jenkins, R. B., Davidson, N. E., Martino, S., Kaufman, P. A., Addo, F. K., Murphy, B., Ingle, J. N., and Perez, E. A. Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. *J. Natl. Cancer Inst. (Bethesda)*, 94: 855–857, 2002.
- Zujewski, J. A. “Build quality in”—HER2 testing in the real world. *J. Natl. Cancer Inst. (Bethesda)*, 94: 788–789, 2002.
- Koscielny, S., Terrier, P., Spielmann, M., and Delarue, J. C. Prognostic importance of low c-erbB2 expression in breast tumors. *J. Natl. Cancer Inst. (Bethesda)*, 90: 712, 1998.
- Koscielny, S., Terrier, P., Daver, A., Wafflart, J., Goussard, J., Ricolleau, G., Delvincourt, C., and Delarue, J. C. Quantitative determination of c-erbB-2 in human breast tumours: potential prognostic significance of low values. *Eur. J. Cancer*, 34: 476–481, 1998.
- Dittadi, R., Brazzale, A., Pappagallo, G., Salbe, C., Nascimben, O., Rosabian, A., and Gion, M. ErbB2 assay in breast cancer: possibly improved clinical information using a quantitative method. *Anticancer Res.*, 17: 1245–1247, 1997.
- Ferrero-Pous, M., Hacene, K., Tubiana-Hulin, M., and Spyrtos, F. Re: Prognostic importance of low c-erbB2 expression in breast tumors. *J. Natl. Cancer Inst. (Bethesda)*, 91: 1584–1585, 1999.
- Ben-Levy, R., Paterson, H. F., Marshall, C. J., and Yarden, Y. A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway. *EMBO J.*, 13: 3302–3311, 1994.
- Amundadottir, L. T., and Leder, P. Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. *Oncogene*, 16: 737–746, 1998.
- Janes, P. W., Daly, R. J., deFazio, A., and Sutherland, R. L. Activation of the Ras signalling pathway in human breast cancer cells overexpressing erbB-2. *Oncogene*, 9: 3601–3608, 1994.
- Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Eystein Lonning, P., and Borresen-Dale, A. L. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. USA*, 98: 10869–10874, 2001.
- Vogel, C. L., Cobleigh, M. A., Tripathy, D., Gutheil, J. C., Harris, L. N., Fehrenbacher, L., Slamon, D. J., Murphy, M., Novotny, W. F., Burchmore, M., Shak, S., Stewart, S. J., and Press, M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.*, 20: 719–726, 2002.
- Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., Wolter, J. M., Paton, V., Shak, S., Lieberman, G., and Slamon, D. J. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.*, 17: 2639–2648, 1999.
- Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.*, 344: 783–792, 2001.
- Seidman, A. D., Fornier, M. N., Esteva, F. J., Tan, L., Kaptain, S., Bach, A., Panageas, K. S., Arroyo, C., Valero, V., Currie, V., Gilewski, T., Theodoulou, M., Moynahan, M. E., Moasser, M., Sklarin, N., Dickler, M., D'Andrea, G., Cristofanilli, M., Rivera, E., Hortobagyi, G. N., Norton, L., and Hudis, C. A. Weekly trastuzumab and paclitaxel therapy for metastatic breast cancer with analysis of efficacy by HER2 immunophenotype and gene amplification. *J. Clin. Oncol.*, 19: 2587–2595, 2001.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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

Robert L. Camp, Marisa Dolled-Filhart, Bonnie L. King, et al.

Cancer Res 2003;63:1445-1448.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/63/7/1445>

Cited articles This article cites 20 articles, 4 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/63/7/1445.full#ref-list-1>

Citing articles This article has been cited by 32 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/63/7/1445.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/63/7/1445>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.