View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by RERO DOC Digital Library

Patterns of HER-2/neu Amplification and Overexpression in Primary and Metastatic Breast Cancer

Ronald Simon, Antonio Nocito, Tanja Hübscher, Christoph Bucher, Joachim Torhorst, Peter Schraml, Lukas Bubendorf, Michael M. Mihatsch, Holger Moch, Kim Wilber, Andreas Schötzau, Juha Kononen, Guido Sauter

Background: **Only 25% of patients with HER-2/neu-positive metastatic breast tumors respond favorably to trastuzamab (Herceptin) treatment. We hypothesized that a high failure rate of patients on trastuzamab could result if some of the metastases were HER-2 negative and these metastases ultimately determine the course of the disease.** ***Methods:*** **We used tissue microarrays (TMAs) containing four samples each from 196 lymph node-negative primary tumors, 196 lymph node-positive primary tumors, and three different lymph node metastases from each lymph node-positive tumor to estimate HER-2 gene amplification by fluorescence *in situ* hybridization (FISH) and Her-2 protein overexpression by immunohistochemistry (IHC).** ***Results:*** **FISH and IHC analyses gave the same result with respect to HER-2 status for 93.7% of the tissues contained in the TMAs. Tissue samples were, therefore, considered to be HER-2 positive if they were positive for either HER-2 DNA amplification or Her-2 protein expression and HER-2 negative if both FISH and IHC gave a negative result. The HER-2 status of lymph node-positive primary tumors was maintained in the majority of their metastases. For HER-2-positive primary tumors, 77% (95% confidence interval [CI] = 59% to 90%) had entirely HER-2-positive metastases, 6.5% (95% CI = 8% to 21%) had entirely HER-2-negative metastases, and 16.3% (95% CI = 5% to 34%) had a mixture of HER-2-positive and HER-2-negative metastases. For HER-2-negative primary tumors, 95% (95% CI = 88% to 98%) had metastases that were entirely negative for HER-2.** ***Conclusions:*** **Our data suggest that differences in HER-2 expression between primary tumors and their lymph node metastases cannot explain the high fraction of nonresponders to trastuzamab therapy. [J Natl Cancer Inst 2001;93:1141–6]**

The HER-2/neu (also known as ERBB2) proto-oncogene, which encodes a transmembrane growth factor receptor with tyrosine kinase activity, has become an important subject for human cancer research during the last decade. The impact of HER-2 amplification and expression on prognosis *(1–12)* and on the response to cytotoxic *(4,13–18)* and hormonal *(15,19–22)* therapies in breast cancer patients have been studied intensively. Studies of HER-2 represent a paradigm of how genetic findings have led to the development of a gene-specific therapy: In September 1998, the U.S. Food and Drug Administration approved trastuzamab (Herceptin), a recombinant monoclonal antibody targeting Her-2, for the treatment of metastatic breast cancer. Although trastuzumab binds to the Her-2 receptor with high affinity, the mechanism of action by which it causes tumor reduction is not understood. Despite the theoretic benefits of such a targeted treatment, not all patients respond favorably to trastuzamab treatment in practice. Among the patients with HER-2-positive metastatic breast cancer that is resistant to conventional cytotoxic treatment, only about 25% benefit from trastuzamab given in combination with cisplatin *(23)*. The genetic features that distinguish the HER-2-positive breast cancers that respond to trastuzamab from those that do not remain unclear. However, it is possible that the extent of HER-2 amplification and/or overexpression in the primary tumor differs from that in the metastases. The HER-2 status of the primary tumor, which is removed from the patient, determines whether or not trastuzamab treatment is prescribed. But trastuzamab works by targeting the metastases that remain in the patient. If at least some of the multiple metastases of an HER-2-positive primary breast tumor did not express HER-2, trastuzamab treatment would most likely not affect the course of the disease. A comprehensive, large-scale study comparing HER-2 gene copy numbers and protein expression in primary tumors and in multiple different metastases derived from them has not been performed. To gain insight into the patterns of HER-2 expression in primary and metastatic breast cancers, we utilized our recently developed tissue microarray (TMA) technique *(24)* to study HER-2 gene amplification and protein overexpression in the primary tumor and in three different metastases of each of 196 highly metastatic breast carcinomas.

Patients and Methods

Patients

We randomly selected two different groups of 196 tumors each from a consecutive series of more than 3000 breast cancers that were routinely examined in the Institute of Pathology at the Kantonsspital Basel (Switzerland) from 1985 through 1995. Each primary tumor came from a different patient. The first group of tumors consisted of 196 lymph node-negative tumors, which included 133 ductal carcinomas, 37 lobular carcinomas, and 26 tumors of other histologic subtypes. Among this group of tumors, 10% were pT1, 74% were pT2, 9% were pT3, and 7% were pT4 carcinomas according to classification by the International Union Against Cancer *(25)*. The second set of tumors consisted of 196 primary breast cancers that had three or more positive axillary lymph nodes with metastases larger than 0.5 cm in diameter and for which tissue blocks were available from both the primary cancer and the axillary lymph node metastases. This group of lymph node-positive primary tumors included 145 ductal carcinomas, 25 lobular carcinomas, and 26 tumors of other histologic subtypes. Among this group of tumors, 14% were pT1, 45% were pT2, 16% were pT3, and 25% were pT4 cancers. We could not determine the tumor stage from the pathology reports for two patients whose tumors were part of this group.

TMA Construction

Tissue samples were fixed in buffered 4% formalin, embedded in paraffin, and used to construct TMAs as described previously *(24)*. Briefly, hematoxylin–eosin-stained sections were made from each selected primary tumor block (donor blocks) to define representative tumor regions. Tissue cylinders (0.6 mm in diameter) were then punched from that region of the donor block with the use of a custom-made precision instrument (Beecher Instruments, Silver Spring, MD).

Tissues cylinders from the 196 lymph node-negative primary tumors, the 196 lymph node-

Affiliations of authors: R. Simon, A. Nocito, T. Hübscher, C. Bucher, J. Torhorst, P. Schraml, L. Bubendorf, M. M. Mihatsch, H. Moch, G. Sauter (Institute of Pathology), A. Schötzau (Eye Clinic), University of Basel, Switzerland; K. Wilber, Vysis Inc., Downers Grove, IL; J. Kononen, Diomeda Life Sciences Inc., Rockville, MD.

Correspondence to: Guido Sauter, M.D., Institute of Pathology, University Hospital, Schoenbeinstrasse 40, 4003 Basel, Switzerland (e-mail: Guido.Sauter@unibas.ch).

See "Notes" following "References."

© Oxford University Press

positive primary tumors, and three different lymph node metastases of each of the lymph node-positive primary tumors (3 × 196 metastases) were distributed among three 25-mm × 35-mm paraffin blocks to produce the TMA blocks used for our study. Two of the blocks in each set contained 400 samples each, and one block contained 180 samples. One complete set of TMA blocks containing a total of 980 tissue samples stained with hematoxylin–eosin is shown in Fig. 1, A; a single tissue spot stained with hematoxylin–eosin-stained is shown in Fig. 1, B.

We constructed four sets of complete TMA blocks; each set contained tissue samples collected from different representative regions of the donor tissues. The resulting TMA blocks were cut into 3-mm sections that were transferred to glass slides by use of the Paraffin Sectioning Aid System (Instrumedics, Hackensack, NJ). A separate section from each of the four complete sets of TMA blocks was used for fluorescence *in situ* hybridization (FISH) and immunohistochemical analysis.

Fluorescence *In Situ* Hybridization

Slides containing sections of TMA blocks were treated before hybridization with a paraffin pretreatment kit (Vysis Inc., Downers Grove, IL) according to the manufacturer's instructions. The sections were air-dried and dehydrated by successive washes in 70%, 85%, and 100% ethanol and then incubated in 70% formamide and 2 × standard saline citrate (SSC) (1.75% sodium chloride/0.89% sodium citrate [pH 7]) for 5 minutes at 74 °C to denature the tumor DNA. Slides were then incubated with a SpectrumOrange™-labeled HER-2 DNA probe and a SpectrumGreen™-labeled centromere 17 reference probe (PathVision™; Vysis Inc.) in hybridization buffer (2 g of dextran sulfate/10 mL of formamide in 2 mL 20 × SSC) overnight at 37 °C in a humidified chamber. After hybridization, the slides were washed in 2 × SSC/0.3% Nonidet P-40, and the DNA was counterstained with 0.2 μ*M* 4,6-diamidino-2-phenylindole (DAPI) in an antifade solution (Vectashield; Vector Laboratories, Inc., Burlingame CA). We analyzed the slides by indirect immunofluorescence microscopy by use of a Zeiss Axiophot microscope equipped with Zeiss filtersets (Carl Zeiss, Göttingen, Germany) for DAPI, fluorescein isothiocyanate, and Texas Red (Carl Zeiss) under 100-fold magnification. In contrast to the manufacturer's directions, we did not count the HER-2 and centromere 17 signals in a defined number of cells for each tumor in this study. Instead, we estimated the mean number of HER-2 and centromere 17 signals for each tumor sample. In previous experiments, we found that estimation of signals and signal counting gave identical results with respect to HER-2 gene amplification *(26)*. Our criteria for HER-2 gene amplification were an HER-2 to centromere 17 signal ratio of at least three or tight clusters of more than five HER-2 signals in more than 10% of tumor cells in the tissue spot. These criteria for HER-2 amplification were similar to those used in previous studies *(27,28)* and resulted in fewer borderline cases than the criterion (an HER-2/centromere 17 signal ratio of at least two) recommended by the manufacturer.

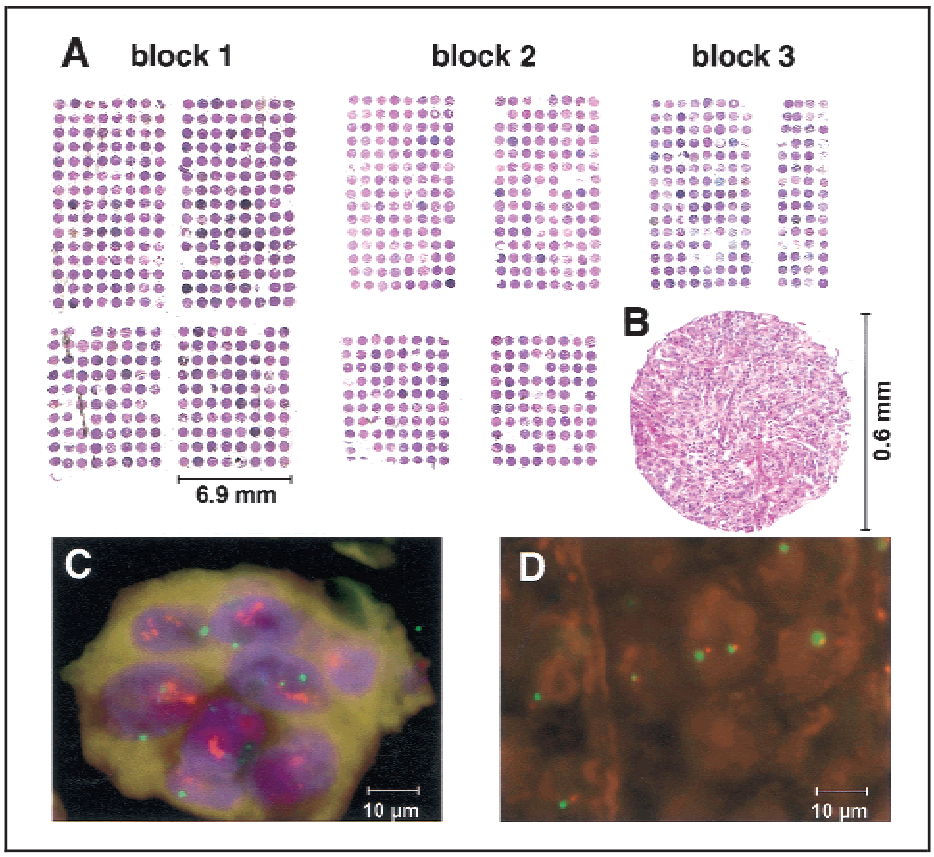

Fig. 1. Breast cancer tissue microarrays (TMAs). **Panel A:** one complete set of TMAs, stained with hematoxylin–eosin, consisting of three paraffin blocks (25 × 30 mm) containing 400 (**block 1**), 400 (**block 2**), and 180 (**block 3**) breast cancer samples. **Panel B:** example of a single hematoxylin–eosin-stained tissue spot. **Panel C:** fluorescence *in situ* hybridization (FISH) analysis of a tumor cell containing amplified HER-2 DNA with two centromere 17 signals (**green**) and a tight cluster containing more than five HER-2 signals (**red**). **Panel C:** FISH analysis of tumor cells containing unamplified HER-2 DNA. Each cell has two centromere 17 signals (**green**) and one or two HER-2 signals (**red**). The nuclear DNA (**blue**) in **panels C** and **D** is stained with 4,6-diamidino-2-phenylindole.

Immunohistochemistry

We analyzed Her-2 protein expression in the TMAs by use of the HercepTest™ kit (DAKO Diagnostics, Glostrup, Denmark) according to the manufacturer's instructions. Briefly, the deparaffinized tissue sections were first incubated in a 95 °C waterbath for 40 minutes to induce epitope retrieval and then at room temperature for 30 minutes with the prediluted primary antibody to Her-2. Bound primary antibody was visualized by use of the dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobins as provided by the manufacturer. We included the positive and negative control cell lines supplied with the HercepTest kit in each immunohistochemistry (IHC) assay to ensure the validity of the staining. Stained TMAs were analyzed by light microscopy by use of a 10× objective. We scored immunohistochemical staining according to the manufacturer's instructions: Tissue samples were classified as positive if they had a score of 2+ or 3+ and as negative if they had a score of 0 or 1+.

Statistical Analysis

We calculated exact 95% confidence intervals (CIs) for proportions based on a binominal probability distribution to assess the accuracy of the estimates of HER2 status in primary tumors and metastases. Fisher's exact test was used to compare the proportions of HER-2-positive and HER-2-negative samples in complete and incomplete datasets to assess the influence of missing values.

Results

We first determined whether FISH and IHC gave the same result with respect to HER-2 status. Because each of the four TMAs contained a tissue sample from a different area of each of the 980 tumor samples, a total of 3920 different samples were analyzed in this study. We could interpret unambiguously the results of the FISH or IHC analyses for 3727 (95.1%) of these 3920 samples. Results of both the FISH and IHC analyses were available for 2857 samples. When we compared the results of the two types of analyses for these 2857 samples, we found that 2677 samples (93.7%) gave the same result with respect to HER-2 status for analyses performed by FISH and by IHC and that these samples showed an increase in the frequency of HER-2 gene amplification

with increasing Her-2 protein expression score (Fig. 2, A). We attributed about two thirds of the uninterpretable results to technical problems associated with the TMAs; these included missing samples (lost during sectioning of the arrays) and tissue samples with low numbers of tumor cells *(28).* Technical problems with FISH, such as weak hybridization signals or excessive background, made the results of other samples uninterpretable. Examples of interpretable tumor tissue samples containing amplified HER-2 DNA and nonamplified HER-2 DNA are shown in Fig. 1, C and D, respectively. On the basis of the strong agreement between FISH and IHC data, we decided to classify a tumor sample as HER-2 positive if at least one of the two tests was interpretable and gave a positive result (i.e., a score of 2+ or 3+ in the IHC assay; HER-2 gene amplification by FISH). All of the remaining interpretable tissues that showed no amplification by FISH and an HER-2 score of 0 or 1+ by IHC were considered to be HER-2 negative.

We next investigated HER-2 status among the four samples of each lymph node-negative primary tumor and of each lymph node-positive primary tumor and of the three metastases corresponding to each lymph node-positive primary tumor. We included in this analysis only the 856 primary tumors and metastases for which all four tissue samples gave an interpretable result (either by FISH or by IHC). As shown in Fig. 2, B, for most of the tumors analyzed, different samples from the same tumor gave the same result with regard to HER-2 status. Overall, among the 856 tissues for which all samples were interpretable, only 23 (2.7%) samples differed between the four tissue samples with respect to HER-2 status.

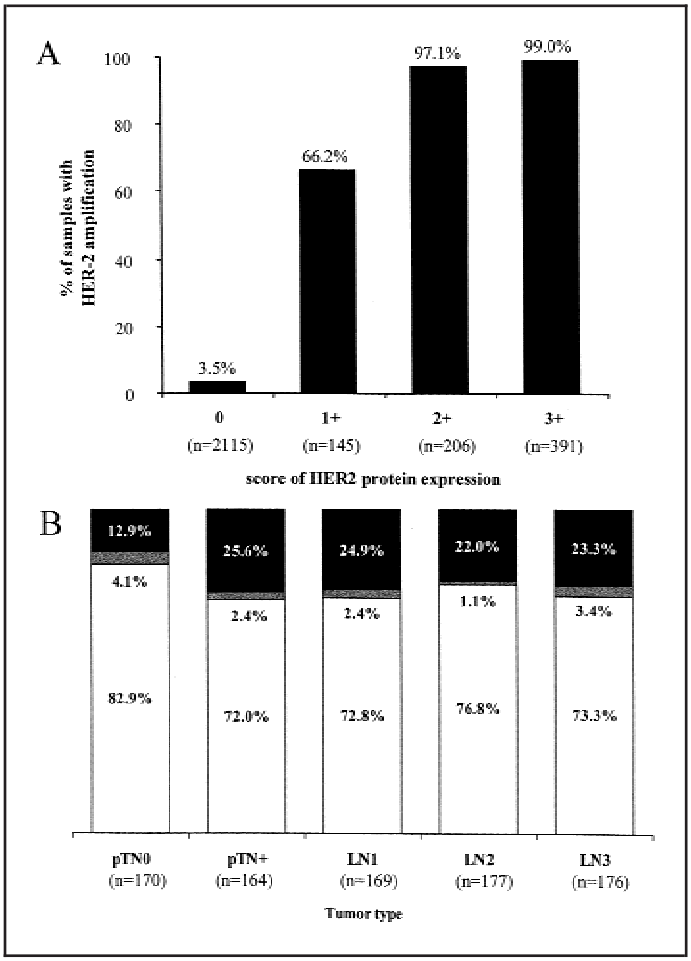

Fig. 2. HER-2 status in different tissue samples from each tumor. **Panel A:** comparison of HER-2 expression determined by immunohistochemistry (IHC) and HER-2 amplification determined by fluorescence *in situ* hybridization (FISH). Only tissue samples from the four replicate arrays that had results for both IHC and FISH (n = 2857) are included. **Bars** represent the fraction (%) of tissues with negative (IHC score of 0), weak (IHC score of 1+), moderate (IHC score of 2+), and strong (IHC score of 3+) Her-2 protein expression that showed HER-2 amplification. **Panel B:** heterogeneity of HER-2 status among the four tissue samples from each tumor. pTN0 = primary lymph node-negative breast cancer; pTN+ = primary lymph node-positive breast cancer; and LN1–LN3 = three sets of lymph node metastases of the pTN+ primary tumors. Only tumors with interpretable immunohistochemical and/or FISH results in all four tissue samples are included. **Black area =** percentage of tumors that were positive for HER-2 (HER-2 gene amplification and/or HercepTest™ score of 2+ or 3+) in all four tissue samples; **white area** = percentage of tumors that were negative for HER-2 (no HER-2 gene amplification and score 0 or 1+) in all four tissue samples; and **gray area** = percentage of tumors for which one or two of the four tissue samples had an HER-2 status that differed from that of the remaining samples.

We then compared the HER-2 status of the lymph node-positive primary tumors with that of their metastases. This analysis was restricted to the 125 lymph node-positive tumors, for which 16 arrayed tissue samples (four samples each of the primary tumor and of three different metastases) yielded interpretable results on HER-2 status. We categorized the primary tumors into one of three groups based on the HER-2 status of the four tissue samples from each tumor; positive, negative, or heterogeneous. Tumors were considered to be positive if all four tissue samples were HER-2 positive, negative if all four tissue samples were HER-2 negative, and heterogeneous if the four tissue samples yielded a mixture of HER-2-positive and HER-2-negative results. On the basis of these criteria, of the 125 lymph node-positive primary tumors, 31 (24.8%; 95% CI = 18% to 33%) were HER-2 positive, 91 (72.8%; 95% CI = 64% to 80%) were HER-2 negative, and three (2.4%; 95% CI = 0.5% to 7%) were heterogeneous for HER-2.

Similarly, for each lymph node-positive primary tumor, we categorized the HER-2 status of each of the associated metastases into one of four groups based on the HER-2 status of the four tissue samples from each metastasis; positive, negative, completely discordant, or partially discordant. The lymph node metastases for a given primary lymph node-positive tumor were considered to be positive if all four tissue samples of each of the three metastases were HER-2 positive, negative if all four tissue samples of each of three metastases were HER-2 negative, completely discordant if all four tissue samples of one or two metastases were either HER-2 positive or HER-2 negative, and partially discordant if at least one metastasis had both HER-2-positive and HER-2-negative tissue samples.

The HER-2 status of the lymph node-positive primary tumors was maintained in the majority of their metastases (Fig. 3). For example, 95% (95% CI = 88% to 98%) of the HER-2-negative primary tumors had metastases that were entirely negative for HER-2, whereas 77% (95% CI = 59% to 90%) of the HER-2-positive primary tumors had metastases that were entirely positive for HER-2. However, a small number of both HER-2-positive and HER-2-negative primary tumors produced metastases whose HER-2 status differed from that of the primary tumor.

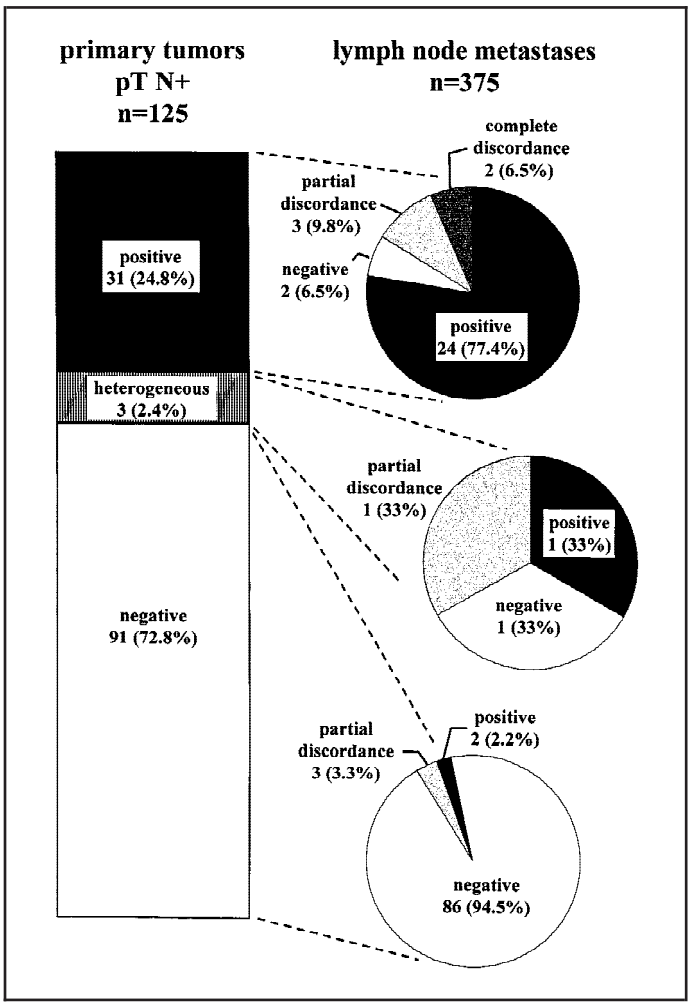

Fig. 3. HER-2 results for a set of 125 primary metastatic breast tumors (pTN+) and 375 (3 × 125) corresponding lymph node metastases. Only tumors for which there were 16 interpretable samples per patient (four tissue samples of each primary tumor and four tissue samples of each of three primary tumor-associated metastases) are included. The column (**left**) shows the fractions of lymph node-positive primary tumors with a positive (all four samples HER-2 positive), negative (all four samples HER-2 negative), and heterogeneous (a mixture of HER-2-positive and HER-2-negative tissue samples) HER-2 status. **Pie charts** show the HER-2 status of the three lymph node metastases associated with the primary lymph node-positive tumors with a given HER-2 status. Negative = all four tissue samples of each of the three primary tumor-associated metastases were HER-2 negative; positive = all four tissue samples of each of the three primary tumor-associated metastases were HER-2 positive; complete discordance = the HER-2 status of all tissue samples of one metastasis differed from the HER-2 status of the remaining metastases; and partial discordance = at least one of the metastases had both positive and negative samples for HER-2.

For example, two (6.5%) of the 31 HER-2-positive primary tumors had metastases that were entirely negative for HER-2, and two (2.2%) of the 91 HER-2-negative primary tumors had metastases that were entirely HER-2 positive. As expected, the three primary tumors with a heterogeneous HER-2 status produced metastases that varied greatly in HER-2 status. One of the heterogeneous primary tumors produced three metastases that were entirely HER-2 positive, one produced three metastases that were entirely HER-2 negative, and one produced metastases that contained both HER-2-positive and HER-2-negative tissue samples.

Of the 125 lymph node-positive primary tumors analyzed, nine (7.2%; 95% CI = 3% to 13%) produced metastases with partially or completely discordant HER-2 status. Only two of these primary tumors (both with amplified HER-2 DNA as determined by FISH) exhibited a complete discordance of HER-2 status (i.e., all samples of at least one of the metastases were HER-2 negative). The remaining seven primary tumors exhibited partial discordance of HER-2 status (i.e., at least one of their metastases had both HER-2-positive and HER-2-negative samples).

To provide a statistical basis for these results and to determine the effects of incomplete datasets on these findings, we calculated exact 95% CIs for proportions to assess the accuracy of our estimates of HER-2 status in the 125 lymph node-positive primary tumors and their metastases. To exclude the possibility that the HER-2 results for the tumors for which not all samples from the four replicate arrays were interpretable might bias these estimates, we also performed a separate calculation for the 68 primary lymph node-positive tumors and their metastases that lacked one or more results for FISH and/or IHC. There was no statistically significant difference ($P = .7866$) in the proportions of HER-2-positive and HER-2-negative tissue samples between complete and incomplete datasets (data not shown).

Discussion

HER-2 is the most frequently overexpressed oncogene in breast cancer: It is detected in approximately 20%–30% of ductal carcinomas. Overexpression of HER-2 is strongly associated with amplification of the HER-2 gene. Although trastuzamab treatment of breast cancer is restricted to patients with metastatic primary tumors that express high amounts of HER-2, this treatment has a high failure rate. We studied HER-2 gene amplification and overexpression in primary tumors and their lymph node metastases to evaluate whether differences in their HER-2 status might account for the failure of trastuzamab treatment. Our results demonstrate that the HER-2 status within individual breast tumors is fairly homogeneous, as is the HER-2 status of primary tumors and their metastases. Fewer than 5% of primary tumors or their metastases showed heterogeneity in HER-2 status.

TMAs containing multiple samples from individual tumor specimens are an ideal tool for a systematic analysis and quantification of tissue heterogeneity. The more heterogeneous the distribution of a particular tissue characteristic, such as HER-2 expression, in a tumor, the less likely it is that the alteration will be detected in all arrayed samples of that tumor. Our observation of little heterogeneity of HER-2 expression in breast cancer is consistent with previous reports describing homogeneous HER-2 expression as assayed by IHC across breast cancer specimens *(29,30).* Further evidence against significant heterogeneity of HER-2 alterations in primary breast cancer is provided by previous studies reporting a high concordance of HER-2 amplification/overexpression between needle biopsy specimens and resected specimens *(26,31).* More than 95% of our patients had a homogeneous HER-2 status in their primary tumor and identical findings in all their metastases. These findings, together with the known high frequency of HER-2 overexpression in ductal carcinoma *in situ (32)* and the lack of a significant increase in HER-2 amplification or overexpression with tumor stage *(33),* suggest that the molecular events that

cause alterations in HER-2 expression occur early in tumorigenesis in a subset of breast cancers. Regardless of the mechanism responsible, cells that overexpress HER-2 may have a strong growth advantage resulting in the rapid replacement of tumor cells that do not overexpress HER-2.

We found evidence for tissue heterogeneity with respect to the proportions of HER-2-positive and HER-2-negative cells detectable by IHC or FISH in a small number of tumors. Among the 91 HER-2-negative primary tumors, five (5.5%) had HER-2-positive metastases. It is unlikely that *de novo* amplification or overexpression of the HER-2 gene occurred in these metastases because, in some of the arrayed samples, all three metastases of the HER-2-negative primary tumors were positive for HER-2. In addition, a small group of heterogeneous primary tumors produced HER-2-positive metastases. These observations suggest that a positive HER-2 status in the primary tumor does not directly influence the metastatic capabilities of tumor cells. In heterogeneous primary tumors having both HER-2-positive and HER-2-negative lymph node metastases, it can be assumed that the molecular event(s) required for metastasis occurred before HER-2 amplification and that separate HER-2-positive and HER-2-negative tumor cells have established the metastases.

The analysis of more than 2500 tumor samples by both IHC and FISH allowed a comprehensive comparison of these two methods of determining HER-2 status. We observed a high concordance (93.7%) between the FISH and the IHC results, which has also been observed in other studies *(34,35).* We observed a high percentage (66%) of HER-2-amplified tumors with a Her-2 protein expression score of 1+. This was unexpected because, in most previous studies, the percentage of HER-2-amplified tumors with HER-2 expression scores of 1+ was less than 10% *(29,36,37).*

The TMA technique has a number of distinct advantages over traditional methods using large sections, including an improved standardization, capacity, and speed of analysis as well as the potential of automatization of both array construction and analysis. By default, the analysis of all tumors of one study under identical conditions in a single experiment optimizes experimental standardization. While tumor blocks containing precious material are usually exhausted after 200–300 sections, the tissue-array technique allows the construction of hundreds of replica arrays from one set of tumors and thus tens of thousands *in situ* analyses. It is likely that the speed and, more important, the objectivity of immunohistochemical analyses will be substantially improved by automated analysis. Further optimizations of the technology, including automation of array analysis, automation of array production, and an increased availability of tissue arrays, will make this approach a standard tool for tissue-based research.

Our data suggest that the high rate of nonresponse to trastuzamab therapy cannot be explained by heterogeneity with respect to HER-2 status between primary tumors and their lymph node metastases. Although we cannot entirely exclude the possibility that very small subpopulations of HER-2-negative cells were present in the HER-2-positive tumors analyzed in our study, we think that it is unlikely that such cells could cause a total lack of response to trastuzamab therapy in patients. An initial treatment effect on the much larger population of HER-2-positive tumor cells would result in a significant tumor reduction that would clearly lead to a clinically detectable remission. Future studies will be needed to identify the genetic or epigenetic differences that may distinguish trastuzamab-responsive from nonresponsive HER-2-positive tumor cells.

REFERENCES

(1) Clark GM, McGuire WL. Follow-up study of HER-2/neu amplification in primary breast cancer. Cancer Res 1991;51:944–8.

(2) Bianchi S, Paglierani M, Zampi G, Cardona G, Cataliotti L, Bonardi R, et al. Prognostic significance of c-erbB-2 expression in node negative breast cancer. Br J Cancer 1993;67:625–9.

(3) Gasparini G, Gullick WJ, Bevilacqua P, Sainsbury JR, Meli S, Boracchi P, et al. Human breast cancer: prognostic significance of the c-erbB-2 oncoprotein compared with epidermal growth factor receptor, DNA ploidy, and conventional pathologic features. J Clin Oncol 1992;10:686–95.

(4) Gusterson BA, Gelber RD, Goldhirsch A, Price KN, Save-Soderborgh J, Anbazhagan R, et al. Prognostic importance of c-erbB-2 expression in breast cancer. International (Ludwig) Breast Cancer Study Group. J Clin Oncol 1992;10:1049–56.

(5) McCann AH, Dervan PA, O'Regan M, Codd MB, Gullick WJ, Tobin BM, et al. Prognostic significance of c-erbB-2 and estrogen receptor status in human breast cancer. Cancer Res 1991;51:3296–303.

(6) Noguchi M, Koyasaki N, Ohta N, Kitagawa H, Earashi M, Thomas M, et al. C-erbB-2 oncoprotein expression versus internal mammary lymph node metastases as additional prognostic factors in patients with axillary lymph node-positive breast cancer. Cancer 1992;69:2953–60.

(7) Quenel N, Wafflart J, Bonichon F, de Mascarel I, Trojani M, Durand M, et al. The prognostic value of c-erbB2 in primary breast carcinomas: a study on 942 cases. Breast Cancer Res Treat 1995;35:283–91.

(8) Rilke F, Colnaghi MI, Cascinelli N, Andreola S, Baldini MT, Bufalino R, et al. Prognostic significance of HER-2/neu expression in breast cancer and its relationship to other prognostic factors. Int J Cancer 1991;49:44–9.

(9) Rosen PP, Lesser ML, Arroyo CD, Cranor M, Borgen P, Norton L. Immunohistochemical detection of HER2/neu in patients with axillary lymph node negative breast carcinoma. A study of epidemiologic risk factors, histologic features, and prognosis. Cancer 1995;75:1320–6.

(10) Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–12.

(11) Thor AD, Berry DA, Budman DR, Muss HB, Kute T, Henderson IC, et al. erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. J Natl Cancer Inst 1998;90:1346–60.

(12) Toikkanen S, Helin H, Isola J, Joensuu H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow-up. J Clin Oncol 1992;10:1044–8.

(13) Tetu B, Brisson J. Prognostic significance of HER-2/neu oncoprotein expression in node-positive breast cancer. The influence of the pattern of immunostaining and adjuvant therapy. Cancer 1994;73:2359–65.

(14) Thor AD, Schwartz LH, Koerner FC, Edgerton SM, Skates SJ, Yin S, et al. Analysis of c-erbB-2 expression in breast carcinomas with clinical follow-up. Cancer Res 1989;49(24 Pt 1):7147–52.

(15) Tetu B, Brisson J, Plante V, Bernard P. p53 and c-erbB-2 as markers of resistance to adjuvant chemotherapy in breast cancer. Mod Pathol 1998;11:823–30.

(16) Stal O, Sullivan S, Wingren S, Skoog L, Rutqvist LE, Carstensen JM, et al. c-erbB-2 expression and benefit from adjuvant chemotherapy and radiotherapy of breast cancer. Eur J Cancer 1995;31A:2185–90.

(17) Muss HB, Thor AD, Berry DA, Kute T, Liu ET, Koerner F, et al. c-erbB-2 expression and response to adjuvant therapy in women with node-positive early breast cancer [published erratum appears in N Engl J Med 1994;331:211]. N Engl J Med 1994;330:1260–6.

(18) Paik S, Bryant J, Park C, Fisher B, Tan-Chiu E, Hyams D, et al. erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J Natl Cancer Inst 1998;90:1361–70.

(19) Borg A, Tandon AK, Sigurdsson H, Clark GM, Ferno M, Fuqua SA, et al. HER-2/neu ampli-

fication predicts poor survival in node-positive breast cancer. Cancer Res 1990;50:4332–7.
(20) Carlomagno C, Perrone F, Gallo C, De Laurentiis M, Lauria R, Morabito A, et al. c-erb B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J Clin Oncol 1996;14:2702–8.
(21) Sjogren S, Inganas M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. J Clin Oncol 1998;16:462–9.
(22) Elledge RM, Green S, Ciocca D, Pugh R, Allred DC, Clark GM, et al. HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. Clin Cancer Res 1998;4:7–12.
(23) Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol 1998;16:2659–71.
(24) Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998; 4:844–7.
(25) International Union Against Cancer. TNM classification of malignant tumors. Berlin (Germany): Springer; 1992.
(26) Sauter G, Feichter G, Torhorst J, Moch H, Novotna H, Wagner U, et al. Fluorescence *in situ* hybridization for detecting erbB-2 amplification in breast tumor fine needle aspiration biopsies. Acta Cytol 1996;40:164–73.
(27) Bubendorf L, Kononen J, Koivisto P, Schraml P, Moch H, Gasser TC, et al. Survey of gene amplifications during prostate cancer progression by high-throughout fluorescence *in situ* hybridization on tissue microarrays. Cancer Res 1999;59:803–6.
(28) Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, et al. Tissue microarrays for gene amplification surveys in many different tumor types. Clin Cancer Res 1999;5: 1966–75.
(29) Jimenez RE, Wallis T, Tabasczka P, Visscher DW. Determination of Her-2/Neu status in breast carcinoma: comparative analysis of immunohistochemistry and fluorescent *in situ* hybridization. Mod Pathol 2000;13:37–45.
(30) Masood S, Bui MM. Assessment of Her-2/neu overexpression in primary breast cancers and their metastatic lesions: an immunohistochemical study. Ann Clin Lab Sci 2000;30: 259–65.
(31) Klijanienko J, Couturier J, Galut M, El-Naggar AK, Maciorowski Z, Padoy E, et al. Detection and quantitation by fluorescence *in situ* hybridization (FISH) and image analysis of HER-2/neu gene amplification in breast cancer fine-needle samples. Cancer 1999;87:312–8.
(32) Allred DC, Clark GM, Molina R, Tandon AK, Schnitt SJ, Gilchrist KW, et al. Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancer. Hum Pathol 1992;23:974–9.
(33) Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S, Watanabe S, et al. Correlation between histologic grade of malignancy and copy number of c-erbB-2 gene in breast carcinoma. A retrospective analysis of 176 cases. Cancer 1990;65:1794–800.
(34) Jacobs TW, Gown AM, Yaziji H, Barnes MJ, Schnitt SJ. Comparison of fluorescence *in situ* hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. J Clin Oncol 1999;17:1974–82.
(35) Bankfalvi A, Simon R, Brandt B, Burger H, Vollmer I, Dockhorn-Dworniczak B, et al. Comparative methodological analysis of erbB-2/HER-2 gene dosage, chromosomal copy number and protein overexpression in breast carcinoma tissues for diagnostic use. Histopathology 2000;37:411–9.
(36) Hoang MP, Sahin AA, Ordonez NG, Sneige N. HER-2/neu gene amplification compared with HER-2/neu protein overexpression and interobserver reproducibility in invasive breast carcinoma. Am J Clin Pathol 2000;113:852–9.
(37) Ridolfi RL, Jamehdor MR, Arber JM. HER-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence *in situ* hybridization approach. Mod Pathol 2000;13: 866–73.

NOTES

Editor's note: Vysis Inc. (Downers Grove, IL) provided the probes for this study. G. Sauter is an advisor to Vysis Inc. and Diomeda Life Sciences Inc. (Rockville, MD). J. Kononen holds stock in Diomeda Life Sciences Inc., and K. Wilber holds stock in Vysis Inc.

We thank Yvonne Knecht, Hedvika Novotny, Martina Mirlacher, Martina Storz, Heidi Oggier, Carole Egenter, and the staff of the Institute of Pathology, University of Basel, Switzerland, for their excellent technical support.

Manuscript received December 27, 2000; revised May 18, 2001; accepted May 30, 2001.