HER2 Heterogeneity Affects Trastuzumab Responses and Survival in Patients With HER2-Positive Metastatic Breast Cancer

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ABSTRACT

Objectives: *Heterogeneity of* HER2 *gene amplification is found in a subset of breast cancers. We investigated the impact of* HER2 *heterogeneity on trastuzumab responses and clinical outcomes in 112 patients with HER2-positive metastatic breast cancer.*

Methods: *Regional and genetic heterogeneity of* HER2 *gene amplification was determined in three different areas of each tumor by immunohistochemistry and silver in situ hybridization. We also assessed the overall levels of* HER2 *amplification and the proportion of tumor cells with a* HER2*/CEP17 ratio of more than 2.2 or strong and complete membranous (3+) expression of HER2 protein.*

Results: HER2 *regional and genetic heterogeneity based on the* HER2*/CEP17 ratio was confirmed in 8.7% and 2.7% of cases, respectively. Poor response to trastuzumab was associated with overall low-level or equivocal amplification,* HER2 *regional heterogeneity by the* HER2*/CEP17 ratio, the* HER2*/CEP17 ratio of more than 2.2 in less than 80% of tumor cells, and HER2 immunohistochemical expression of 3+ in less than 75% of tumor cells. In survival analyses, low-level or equivocal* HER2 *amplification,* HER2 *regional heterogeneity based on the* HER2*/CEP17 ratio, and the* HER2*/CEP17 ratio of more than 2.2 in less than 80% of tumor cells were associated with shorter time to progression and lower overall survival in univariate and multivariate analyses.*

Conclusions: *These results suggest that accurate assessment of* HER2 *status, including* HER2 *heterogeneity, is important in predicting trastuzumab responses and outcomes in patients with HER2-positive metastatic breast cancer.*

CME/SAM

Upon completion of this activity you will be able to:
- describe *HER2* genetic heterogeneity defined by College of American Pathologists guidelines.
- predict the cases that are likely to have *HER2* heterogeneity.
- define the characteristics of HER2-positive breast cancers associated with poor response to trastuzumab.
- list the information that should be included in *HER2* in situ hybridization reports.

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Questions appear on p 877. Exam is located at www.ascp.org/ajcpcme.

Overexpression of human epidermal growth factor receptor 2 (HER2), usually associated with amplification of the *HER2* gene, has been identified in 15% to 20% of breast cancers and is associated with an aggressive clinical behavior and poor prognosis.[1-3] Since the introduction of trastuzumab, a humanized monoclonal antibody against the extracellular portion of the HER2 protein, treatment outcomes have improved substantially. Trastuzumab has been demonstrated to be effective in patients with HER2-postive metastatic breast cancer and, as adjuvant treatment, in patients with operable HER2-positive breast cancer.[4-9] In combination with chemotherapy, trastuzumab was shown to significantly improve objective response rates, time to progression, and overall survival in patients with HER2-positive metastatic breast cancer.[5] Notwithstanding its clinical benefit, cases of primary and secondary resistance to trastuzumab have been frequent.[4-9] Some of the possible explanatory mechanisms include impaired access of trastuzumab to HER2, alternative

signaling from the insulin-like growth factor 1 receptor or other epidermal growth factor receptors, aberrant activation of downstream signaling via loss of PTEN (phosphatase and tensin homolog deleted on chromosome 10), and downregulation of p27.[10,11] However, the status of *HER2*, and especially of *HER2* heterogeneity, has not been well studied as a potential factor in trastuzumab resistance.

Tumors showing low-level *HER2* gene amplification or equivocal HER2 protein expression may show a decreased response to trastuzumab. Patients with metastatic breast cancer with a score of 2+ for the HER2 protein expression were less responsive to trastuzumab than those with a score of 3+.[5,7] Previously, we showed that low-level *HER2* amplification was associated with decreased time to progression compared with high-level amplification in patients with metastatic breast cancer treated with weekly paclitaxel and trastuzumab.[12] Furthermore, in a neoadjuvant setting, low-level *HER2* amplification correlated with a lower rate of pathologic response to trastuzumab-based therapy.[13]

HER2 heterogeneity has been found in a subset of breast cancers, in which it has been linked to low-level *HER2* amplification or equivocal HER2 expression.[14-17] Its importance is increasingly being recognized not only in breast cancer but also in other cancers, including gastric[18] and esophageal adenocarcinoma.[19] While *HER2* heterogeneity may contribute to inaccurate assessment of *HER2* status and affect treatment decisions, more important, it could also attenuate the response to HER2-targeted therapy. However, the evidence in this area is not yet clear.

In the present study, we investigated the impact of *HER2* heterogeneity on trastuzumab responses and clinical outcomes in patients with HER2-positive metastatic breast cancer who had received trastuzumab-based chemotherapy. We evaluated two aspects of *HER2* heterogeneity: (1) regional heterogeneity, defined as the existence of amplification-negative or amplification-equivocal patterns in different areas of the tumor, and (2) genetic heterogeneity, defined as the presence of tumor cells with a *HER2*/CEP17 ratio higher than 2.2 (or >6 *HER2* signals) in 5% to 50% of tumor cells.[20] We also determined the overall levels of *HER2* gene amplification, as well as the frequency of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 and with an immunohistochemistry (IHC) score of 3+, and measured the correlations of these factors with trastuzumab responses and survival.

Materials and Methods

Patients and Tissue Specimens

We retrospectively reviewed the records of 112 patients with HER2-positive metastatic breast cancer treated with trastuzumab-based chemotherapy at the Seoul National University Bundang Hospital, the Seoul National University Hospital, and the Asan Medical Center from January 2004 to December 2011. Eligibility criteria included HER2 positivity demonstrated by IHC 3+ and/or fluorescence in situ hybridization (FISH) or silver in situ hybridization (SISH); availability of formalin-fixed, paraffin-embedded tissue of the primary or metastatic carcinoma; trastuzumab-based treatment after a diagnosis of metastatic disease; and no previous trastuzumab therapy.

Clinicopathologic data were obtained from the medical records and from the available H&E-stained sections. We also determined the following variables: histologic subtype, T stage, N stage, Bloom-Richardson histologic grade, estrogen receptor status, and progesterone receptor status. All cases were independently reviewed by two breast pathologists (S.Y.P. and H.J.L.). The study was approved by the institutional review board (IRB) of each institution (protocol B-1302/190-301, IRB of Seoul National University Bundang Hospital; protocol H-1303-069-474, IRB of Seoul National University Hospital; protocol S2012-1344-0002, IRB of Asan Medical Center), waiving the requirement for informed consent for the study. This study was performed in accordance with the Declaration of Helsinki.

Immunohistochemical Evaluation of HER2 Expression

HER2 protein expression had been determined by using two different antibodies (rabbit polyclonal antibody A0485 from DAKO [Carpinteria, CA] and mouse monoclonal antibody CB11 from Novocastra [Newcastle upon Tyne, England]) at the three participating hospitals. For the purpose of this study, HER2 expression was reassessed. Formalin-fixed, paraffin-embedded representative tissue samples were cut into 4-μm sections, dried, deparaffinized, and rehydrated following standard procedures. Subsequently, all sections underwent heat-induced antigen retrieval. Immunohistochemical staining for HER2 (4B5; Ventana Medical Systems, Tucson, AZ) was carried out using ultraView Universal DAB detection kits (Ventana Medical Systems) on the BenchMark XT autostainer (Ventana Medical Systems). Expression of HER2 was scored according to the 2007 American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines: 0, no staining; 1+, weak and incomplete membranous staining in 10% or more of the tumor cells; 2+, weak-to-moderate, complete membranous staining in 10% or more of the tumor cells; and 3+, strong, complete membranous staining in 30% or more of the tumor cells.[21] The proportion of cells with strong and complete membranous expression (score of 3+) was also recorded. After whole-section screening, three representative areas were selected for separate evaluation of HER2 expression. If the tumor showed regional differences in HER2 expression, differentially stained areas were selected.

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SISH Assays for *HER2* Gene Amplification

Serial 4-μm tissue sections were prepared from each tumor. Automated SISH assays were performed with INFORM HER2 DNA and Chromosome 17 probes (Ventana Medical Systems) using an ultraView SISH Detection Kit (Ventana Medical Systems) according to the manufacturer's protocols.[22] Both probes are prelabeled with dinitrophenol (DNP). The HER2 DNA probe was denatured at 80°C for 12 minutes, and hybridization was performed at 44°C for 6 hours, followed by appropriate stringency washes (three times at 72°C). The chromosome 17 probe was denatured at 80°C for 12 minutes, and hybridization was performed at 44°C for 6 hours on the same slide, followed by further stringency washes (three times at 72°C). The probes were visualized using a rabbit anti-DNP primary antibody and goat anti–rabbit antibody conjugated to horseradish peroxidase as the chromogenic enzyme. The silver precipitate was deposited in the nuclei after sequential addition of silver acetate, hydroquinone, and H_2O_2, and a single copy of the *HER2* gene was seen as a black dot. A red dot for chromosome 17 appeared following a reaction with fast red and naphthol phosphate. The specimens were then counterstained with Harris hematoxylin.

We assessed *HER2* gene amplification status in the three areas that were selected for the evaluation of HER2 protein expression. Fifty cells were evaluated in each area, with a total of 150 cells counted for each case where possible. The genetic variables reported included *HER2* gene copy number, chromosome 17 copy number, and ratio of *HER2* gene to chromosome 17. In cases with distinct subpopulations of amplified and nonamplified cells, counting was weighted by the percentage of each population in the entire tumor. Overall *HER2* amplification was determined based on the ratio defined by the 2007 ASCO/CAP criteria.[21] The *HER2* gene was considered amplified if the average ratio of *HER2* to CEP17 signals was more than 2.2. A ratio of 4.0 or more was considered high-level amplification; a ratio of more than 2.2 and less than 4.0 was considered low-level amplification. Cases with a ratio of 1.8 or higher and 2.2 or lower were considered equivocal for amplification. The cutoff values for chromosome 17 copy number changes were adopted from Ma et al[23] with modifications. Specimens with signals in the range of 1.25 to 2.25 were defined as having disomy 17. The remaining cases were aneusomic for chromosome 17—that is, they had monosomy 17 (<1.25 signals per cells), low polysomy 17 (>2.25 but ≤3.75 signals per cell), or high polysomy 17 (>3.75 signals per cell).

The presence of intratumoral heterogeneity of *HER2* amplification was confirmed by two different methods, as previously described.[17] First, if the assays gave discordant results across the three areas (ie, if there were amplification-negative and amplification-equivocal patterns in different areas), the case was considered to have *HER2* regional heterogeneity. Second, *HER2* genetic heterogeneity was defined, according to the CAP guidelines, as the existence of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 (or >6 *HER2* signals) in 5% to 50% of all examined tumor cells.[20]

Assessment of Trastuzumab Response and Statistical Analysis

The response to trastuzumab-based therapy had been evaluated every 8 to 12 weeks using Response Evaluation Criteria in Solid Tumors version 1.1.[24] Clinical benefit was defined as complete response, partial response, or stable disease for at least 6 months. Time to progression (TTP) was defined as the time from initiation of trastuzumab treatment to disease progression and overall survival (OS) as the time from initiation of trastuzumab treatment to death from any cause.

Data were analyzed using SPSS software version 18.0 for Windows (SPSS, Chicago, IL). The relationship between *HER2* status and trastuzumab response and the clinicopathologic characteristics of the tumors were assessed using the Fisher exact test or the χ^2 test. Receiver operating characteristic (ROC) curve analyses were performed to identify the most appropriate cutoff value for the proportions of cells with a *HER2*/CEP17 ratio of more than 2.2 and with a HER2 IHC of 3+ and *HER2* gene copy number, which were associated with the maximum clinical benefit. Survival curves were estimated using the Kaplan-Meier method and compared using the log-rank test. Covariates that were statistically significant in a univariate model were included in a multivariate analysis using the Cox proportional hazards regression model. Hazard ratios and their 95% confidence intervals were estimated for all factors. *P* values less than .05 were considered statistically significant. All *P* values are two-sided.

Results

Clinicopathologic Characteristics of the Study Population

Clinicopathologic characteristics of the 112 patients are summarized in **Table 1**. Forty-two cases presented as metastatic breast cancer from the outset, and 70 patients were initially diagnosed with early breast cancer followed by curative resection and then progressed to metastatic disease. In 80 of the 112 cases, the tissue originated from the primary site, and in the remaining cases, it was obtained from metastatic sites. Trastuzumab was administered as the first-line treatment for metastatic breast cancer in 99 (88.4%) patients. Among the agents used in trastuzumab-based combination chemotherapy, taxane was used most frequently (in 107 [95.5%] patients). Other agents (ie, gemcitabine, capecitabine, and vinorelbine) were combined with trastuzumab in three cases. Complete

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Table 1
Clinicopathologic Characteristics of 112 Patients With HER2-Positive Metastatic Breast Cancer[a]

Clinicopathologic Characteristic	Value
Age at primary diagnosis, y	
Median	49
Range	27-82
Performance status	
0 or 1	102 (91.1)
2	10 (8.9)
Histology	
Ductal	109 (97.3)
Other	3 (2.7)
Histologic grade	
II	42 (37.5)
III	70 (62.5)
Hormonal status in primary site	
ER+/PR+	30 (26.8)
ER+/PR–	16 (14.3)
ER–/PR–	64 (57.1)
ER–/PR+	2 (1.8)
Overall HER2 immunohistochemistry	
1+ or 2+	12 (10.7)
3+	100 (89.3)
Timing of metastasis diagnosis	
At presentation	42 (37.5)
At recurrence	70 (62.5)
Metastatic location	
Regional	17 (15.2)
Distant	95 (84.8)
No. of metastatic sites	
<3	87 (77.7)
≥3	25 (22.3)
Metastatic sites	
Liver	27 (24.1)
Lung	52 (46.4)
Bone	51 (45.5)
Brain	8 (7.1)
Lymph node	49 (43.8)
Others	7 (6.2)
History of adjuvant chemotherapy	
Anthracycline based	31 (27.7)
Anthracycline and taxane based	20 (17.9)
CMF	10 (8.9)
History of adjuvant hormone therapy	23 (20.5)
History of adjuvant radiotherapy	29 (25.9)
Trastuzumab therapy	
Alone	2 (1.8)
With chemotherapy	
Taxanes	107 (95.5)
Others	3 (2.7)
Lines of trastuzumab therapy	
First line	99 (88.4)
Second line	11 (9.8)
≥Third line	2 (1.8)

CMF, cyclophosphamide, methotrexate, fluorouracil; ER+, estrogen receptor positive; ER–, estrogen receptor negative; PR+, progesterone receptor positive; PR–, progesterone receptor negative.
[a] Values are presented as number (%) unless otherwise indicated.

response and partial response were achieved in nine (8.0%) and 76 (67.9%) patients, respectively. Stable disease was the best outcome in 15 (13.4%) patients with various periods (range, 4.1-18.2 months), and clinical benefit was observed in 91 (81.3%) patients. Eight (7.1%) patients showed progression of the disease, and the response to treatment was not assessable in four (3.6%). The median TTP was 11.0 months (range, 1.3-84.6 months), and the median OS was 32.1 months (range, 1.4-90.1 months) for all patients.

HER2 Amplification and Intratumoral *HER2* Heterogeneity Based on the *HER2*/CEP17 Ratio

The 112 cases were composed of 86 (76.8%) cases with high-level amplification, 23 (20.5%) with low-level amplification, and three (2.7%) with equivocal amplification by SISH. Of 86 cases with high-level amplification, 79 had two or three assessable areas: 69 had high-level amplification and 10 had high/low-level amplification in different areas. Of the 23 cases with low-level amplification, 21 had two or three assessable areas: four showed high/low-level amplification, one showed high/low-level/negative for amplification, one had high-level/equivocal/negative for amplification, 11 had low-level amplification, and four had low-level/equivocal for amplification results in the different tissue areas. Three cases with equivocal *HER2* amplification showed two low-level/equivocal for amplification and one low-level/equivocal/negative for amplification patterns across the three tissue areas. Thus, of the 103 cases with two or three assessable areas, nine (8.7%) had *HER2* regional heterogeneity based on the *HER2*/CEP17 ratio **Table 2** and **Image 1**.

For each case, we assessed the proportion of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 and found that it ranged from 30% to 100% (mean, 87.8%). *HER2* genetic heterogeneity by the *HER2*/CEP17 ratio was confirmed in three (2.7%) cases, all of which showed *HER2* regional heterogeneity. The relationship between *HER2* heterogeneity and various clinicopathologic parameters was also assessed (Supplementary Table 1, available at http://www.ascp.org/docs/default-source/pdf/press/leedec14.pdf). *HER2* heterogeneity was associated with low-level or equivocal *HER2* gene amplification and 1+ or 2+ HER2 protein expression. In addition, the proportion of tumor cells with an IHC score of 3+ and cells with a *HER2*/CEP17 ratio of more than 2.2 was significantly lower in cases with *HER2* regional and genetic heterogeneity.

HER2 protein expression was evaluated in the entire section as well as the three selected areas. Of the 103 cases with two or three assessable areas, 85 patients had the same IHC scores in the different areas: 83 were scored as 3+ and two were scored as 2+. In the remaining 18 cases, HER2 expression varied across two or three areas: the scores were 3+/2+ in six cases, 3+/2+/1+ in one case, 3+/2+/0 in one case, 3+/1+ in two cases, 2+/1+ in seven cases, and 2+/1+/0 in one case. Six of the 18 cases with discordant IHC results across different tumor areas showed *HER2* regional heterogeneity based on the *HER2*/CEP17 ratio.

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Table 2
Intratumoral Heterogeneity of *HER2* Gene Amplification Based on the *HER2*/CEP17 Ratio

Pattern of *HER2* Regional Heterogeneity	Case No.	*HER2*/CEP17 Ratio				HER2 Immunohistochemistry Score					*HER2* Genetic Heterogeneity[a]	% of Cells With *HER2*/CEP17 Ratio >2.2
		Area 1	Area 2	Area 3	Overall	Area 1	Area 2	Area 3	Overall	3+ Percent		
High-level/low-level/negative for amplification	42	3.14	1.14	5.41	3.29	2+	0	3+	2+	10	0	52.7
High-level/equivocal/negative for amplification	104	8.54	2.13	1.68	3.94	3+	1+	1+	2+	10	0	56.0
Low-level/equivocal for amplification	24	2.32	2.40	2.17	2.32	1+	2+	2+	2+	0	0	52.1
	32	2.19	2.59	2.83	2.52	3+	3+	3+	3+	70	0	53.3
	51	1.98	1.93	2.35	2.08	3+	3+	3+	3+	90	1	44.0
	60	2.20	3.22	3.83	3.06	3+	3+	3+	3+	90	0	73.3
	83	2.25	2.42	2.17	2.27	2+	1+	1+	2+	0	1	48.0
	112	2.15	2.39	1.96	2.17	1+	2+	1+	2+	0	0	51.3
Low-level/equivocal/negative for amplification	53	2.39	1.74	1.96	2.02	2+	1+	1+	2+	5	1	30.0

[a] 0, absent; 1, present.

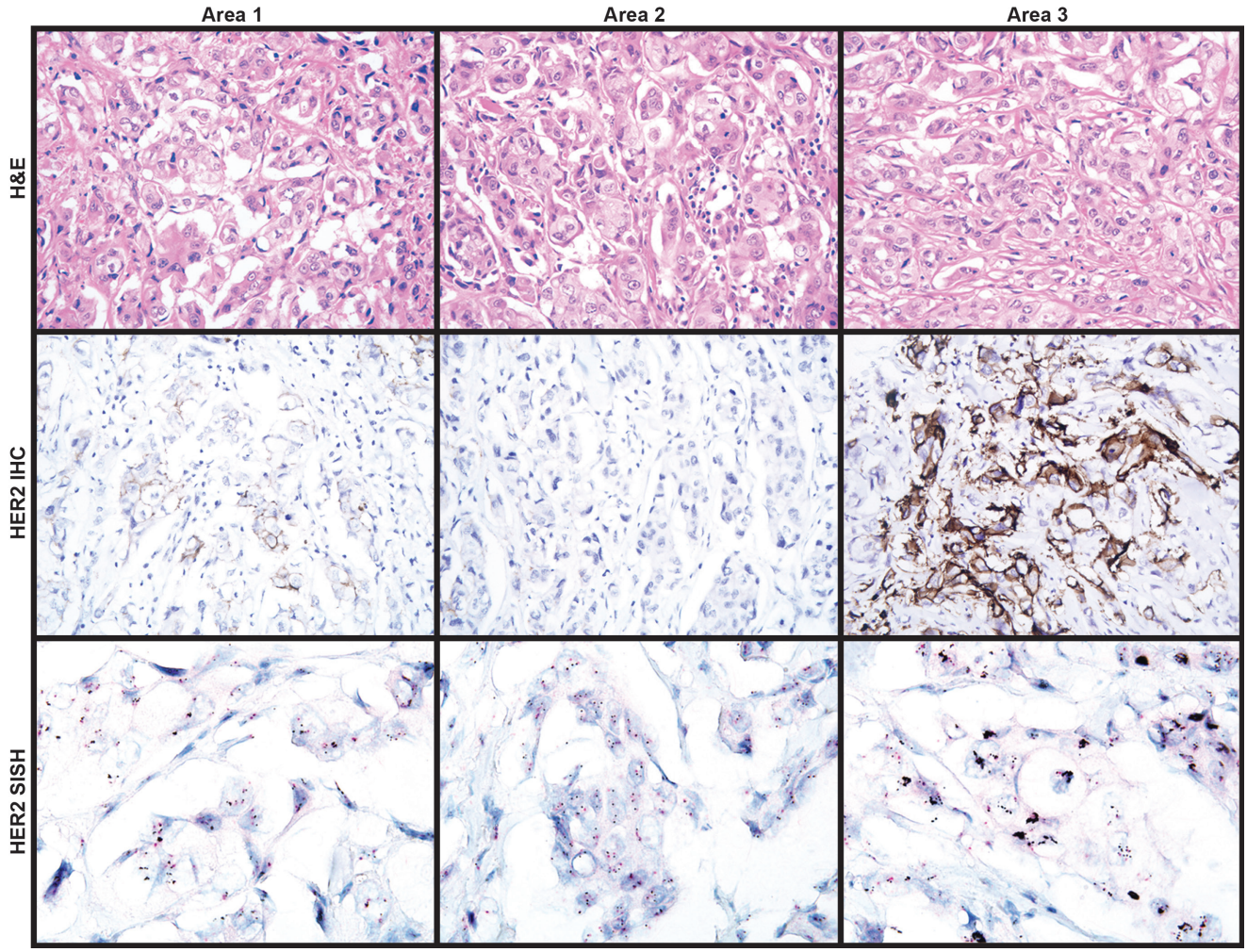

Image 1 Regional heterogeneity of *HER2* gene amplification. An invasive ductal carcinoma (case 42) showing heterogeneous HER2 expression across three tumor areas (2+ in area 1, 0 in area 2, and 3+ in area 3). *HER2* amplification was confined to areas 1 and 3 (H&E, ×200; IHC, ×200; SISH, ×400). IHC, immunohistochemistry; SISH, silver in situ hybridization.

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Table 3
Intratumoral Heterogeneity of *HER2* Gene Amplification by *HER2* Gene Copy Number

		HER2 Gene Copy Number				HER2 Immunohistochemistry Score						
Pattern of *HER2* Regional Heterogeneity	Case No.	Area 1	Area 2	Area 3	Overall	Area 1	Area 2	Area 3	Overall	3+ Percent	*HER2* Genetic Heterogeneity[a]	% of Cells With *HER2* Gene Copy Number>6
Amplification/negative for amplification	42	8.16	2.08	10.92	7.05	2+	0	3+	2+	10	1	48.0
Amplification/equivocal for amplification	104	17.60	4.42	4.28	8.77	3+	1+	1+	2+	10	1	16.0
	6	5.38	6.44	5.36	5.65	3+	3+	3+	3+	90	1	34.6
	31	6.98	5.58	8.02	6.86	3+	3+	3+	3+	100	1	47.3
	61	6.24	5.82	6.98	6.35	3+	3+	3+	3+	100	1	47.3
	82	5.48	6.02	7.42	6.31	1+	1+	2+	3+	30	1	44.7
	93	4.79	5.38	6.16	5.50	2+	2+	2+	2+	5	1	27.5
	108	6.90	7.60	5.94	6.81	3+	3+	3+	3+	90	1	46.7
	109	4.90	6.12	6.46	5.83	3+	3+	3+	3+	70	1	32.0
	112	5.30	6.26	4.90	5.49	1+	2+	1+	2+	0	0	4.2

[a] 0, absent; 1, present.

Intratumoral *HER2* Heterogeneity by *HER2* Gene Copy Number

We also evaluated *HER2* heterogeneity using only the *HER2* gene copy number. The *HER2* gene was considered amplified if the average *HER2* gene copy was more than 6 and not amplified if the average HER2 gene copy was less than 4 by the 2007 ASCO/CAP criteria.[21] The cases with a *HER2* gene copy number of 4 or more and 6 or less were considered equivocal for amplification.[21] Using these criteria, *HER2* regional heterogeneity was found in 10 (9.7%) of the 103 cases with two or three assessable areas **Table 3**. *HER2* genetic heterogeneity, defined as the presence of tumor cells with a *HER2* gene copy number higher than 6 in 5% to 50% of tumor cells, was confirmed in 18 (16.1%) cases, nine of which showed regional heterogeneity (Table 3). Of the 10 cases with *HER2* regional heterogeneity by the *HER2* gene copy number, three also showed *HER2* regional heterogeneity by the *HER2*/CEP17 ratio.

Correlation Between *HER2* Status and Response to Treatment

We evaluated the relationship between *HER2* status and response to trastuzumab treatment. **Table 4** shows the clinical benefit and objective response rates for trastuzumab-based therapy according to *HER2* status. Low-level or equivocal *HER2* amplification and *HER2* regional heterogeneity by the *HER2*/CEP17 ratio were significantly correlated with poor response to trastuzumab ($P = .016$ and $P = .022$ for clinical benefit; $P = .001$ and $P = .013$ for objective response, respectively). However, *HER2* genetic heterogeneity by the *HER2*/CEP17 ratio and *HER2* regional heterogeneity by the *HER2* gene copy number were not correlated with response to trastuzumab. *HER2* genetic heterogeneity based on the *HER2* gene copy number was associated with poor objective response to trastuzumab ($P = .023$). The ROC curve analyses showed that cutoff values of 80% for the proportion of tumor cells with a *HER2*/CEP17 ratio of more than 2.2, 75% for the proportion of tumor cells with an IHC score of 3+, and a *HER2* gene copy number of 12 maximized the sum of sensitivity and specificity in predicting the clinical benefit of trastuzumab. Cases with fewer than 80% of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 and fewer than 75% of tumor cells with a HER2 IHC score of 3+ had significantly poorer responses to trastuzumab-based chemotherapy ($P = .021$ and $P = .027$ for clinical benefit; $P = .002$ and $P = .011$ for objective response, respectively). *HER2* gene copy number less than 12 was significantly correlated with poor objective response to trastuzumab ($P = .019$) but not with clinical benefit.

HER2 Status and Clinical Outcomes

We also investigated how the clinical outcomes were associated with various clinicopathologic parameters, including *HER2* status **Table 5** and **Figure 1**. In a univariate analysis, cases with low-level or equivocal *HER2* gene amplification had significantly shorter TTP (hazard ratio [HR], 2.203; $P = .002$) and OS (HR, 2.608; $P = .001$). Likewise, the presence of *HER2* regional heterogeneity based on the *HER2*/CEP17 ratio was significantly associated with shorter TTP (HR, 2.515; $P = .015$) and OS (HR, 2.888; $P = .015$). Although *HER2* genetic heterogeneity by the *HER2*/CEP17 ratio was not associated with TTP or OS, the proportion of cells with a *HER2*/CEP17 ratio of more than 2.2 was associated with TTP (<80% vs ≥80%; HR, 1.737; $P = .033$) and OS (HR, 2.237; $P = .007$). The proportion of tumor cells with a HER2 IHC score of 3+ was associated with TTP (<75% vs ≥75%; HR, 1.769; $P = .031$) but not with OS. *HER2* regional and genetic heterogeneity by *HER2* gene copy number was associated with OS (HR, 2.421; $P = .021$; HR, 2.011; $P = .031$, respectively) but not with TTP. Other factors associated with TTP and OS included number of metastatic sites (≥3 vs <3; TTP: HR, 1.845; $P = .009$; OS: HR, 2.378; $P = .002$) and performance status (2 vs 0 or 1; TTP: HR, 1.422; $P = .036$;

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Table 4
Clinical Response to Trastuzumab According to HER2 Status

Variable	Clinical Benefit		Objective Response	
	No. (%)	*P* Value	No. (%)	*P* Value
Overall *HER2* gene amplification				
High	74 (89.2)	.016	72 (86.7)	.001
Low or equivocal	17 (68.0)		13 (52.0)	
HER2 regional heterogeneity by *HER2*/CEP17 ratio				
Absent	79 (86.8)	.022	74 (81.3)	.013
Present	4 (50.0)		3 (37.5)	
HER2 genetic heterogeneity by *HER2*/CEP17 ratio				
Absent	88 (83.8)	1	83 (79.0)	1
Present	3 (100.0)		2 (66.7)	
HER2 regional heterogeneity by *HER2* gene copy number				
Absent	84 (84.8)	.631	79 (79.8)	.398
Present	7 (77.8)		6 (66.7)	
HER2 genetic heterogeneity by *HER2* gene copy number				
Absent	78 (86.7)	.155	75 (83.3)	.023
Present	13 (72.2)		10 (55.6)	
Percentage of cells with a *HER2*/CEP17 ratio >2.2				
<80%	14 (66.7)	.021	11 (52.4)	.002
≥80%	77 (88.5)		74 (85.1)	
Percentage of cells with HER2 3+ expression				
<75%	12 (66.7)	.027	10 (55.6)	.011
≥75%	79 (88.8)		75 (84.3)	
HER2 gene copy number				
<12	39 (76.5)	.062	35 (68.6)	.019
≥12	52 (91.2)		50 (87.7)	
Overall HER2 immunohistochemical staining score				
1+ or 2+	8 (72.7)	.374	6 (54.5)	.054
3+	83 (85.6)		79 (81.4)	

OS: HR, 1.528; $P = .036$). *HER2* gene copy number (≥12 vs <12), overall HER2 IHC staining score, chromosome 17 polysomy, hormone receptor status, and age were not correlated with either TTP or OS.

Factors such as degree of *HER2* amplification, *HER2* regional heterogeneity by the *HER2*/CEP17 ratio, proportion of tumor cells with a *HER2*/CEP17 ratio of more than 2.2, and proportion of tumor cells with a HER2 expression score of 3+ were significantly correlated with each other ($r > 0.410$, $P < .001$), and multivariate analyses were performed in different models **Table 6**. Independent negative predictive factors of TTP identified in multivariate analyses included low or equivocal *HER2* gene amplification (HR, 2.284; 95% CI, 1.397-3.735; $P = .001$), *HER2* regional heterogeneity by the *HER2*/CEP17 ratio (HR, 2.475; 95% CI, 1.169-5.242; $P = .018$), less than 80% of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 (HR, 1.827; 95% CI, 1.091-3.058; $P = .022$), and less than 75% of tumor cells with a HER2 expression score of 3+ (HR, 2.101; 95% CI, 1.233-3.580; $P = .006$). Independent predictive factors for poor OS in multivariate analyses included low or equivocal *HER2* gene amplification (HR, 2.830; 95% CI, 1.606-4.986; $P < .001$), *HER2* regional heterogeneity by the *HER2*/CEP17 ratio (HR, 3.509; 95% CI, 1.461-8.426; $P = .005$), and less than 80% of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 (HR, 2.498; 95% CI, 1.372-4.549; $P = .003$). The number of metastatic sites and performance status were independent predictors of TTP and OS (Table 6).

Discussion

In the present study, we showed that *HER2* heterogeneity, assessed using a series of variables—including levels of amplification, regional heterogeneity based on the *HER2*/CEP17 ratio, and proportion of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 or with a HER2 IHC score of 3+—was significantly correlated with the response to trastuzumab treatment and clinical outcomes in patients with HER2-positive metastatic breast cancer who received trastuzumab-based chemotherapy. To our knowledge, this is the first study investigating the impact of *HER2* heterogeneity on the treatment outcomes of HER2-targeted therapy.

Previously, *HER2* status had been considered relatively homogeneous across all cells within a tumor and constant during the progression of breast cancer, suggesting that anti-HER2 therapy would successfully target most of the tumor cells in patients with HER2-positive breast cancer.[10] However, there is increasing recognition of intratumoral heterogeneity of HER2 expression and *HER2* amplification in a significant

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Table 5
Time to Progression and Overall Survival in Univariate Analyses

Variable	Time to Progression		Overall Survival	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Overall *HER2* gene amplification				
High	—		—	
Low or equivocal	2.203 (1.369-3.544)	.002	2.608 (1.502-4.529)	.001
HER2 regional heterogeneity by *HER2*/CEP17 ratio				
Absent	—		—	
Present	2.515 (1.199-5.279)	.015	2.888 (1.225-6.813)	.015
HER2 genetic heterogeneity by *HER2*/CEP17 ratio				
Absent	—		—	
Present	1.288 (0.406-4.085)	.667	1.907 (0.264-13.795)	.523
HER2 regional heterogeneity by *HER2* gene copy number				
Absent	—		—	
Present	1.714 (0.855-3.439)	.129	2.421 (1.145-5.118)	.021
HER2 genetic heterogeneity by *HER2* gene copy number				
Absent	—		—	
Present	1.694 (0.987-2.905)	.056	2.011 (1.067-3.792)	.031
Percentage of cells with a *HER2*/CEP17 ratio >2.2				
≥80%	—		—	
<80%	1.737 (1.045-2.886)	.033	2.237 (1.240-4.035)	.007
Percentage of cells with strong and complete membranous HER2 expression				
≥75%	—		—	
<75%	1.769 (1.054-2.971)	.031	1.274 (0.662-2.455)	.468
HER2 gene copy number				
≥12	—		—	
<12	1.316 (0.883-1.961)	.177	1.473 (0.887-2.447)	.135
Overall HER2 immunohistochemical staining score				
3+	—		—	
1+ or 2+	1.596 (0.868-2.935)	.132	1.199 (0.545-2.637)	.652
Chromosome 17 polysomy				
Absent	—		—	
Present	1.034 (0.655-1.632)	.885	1.458 (0.836-2.543)	.184
Hormone receptor				
Present	—		—	
Absent	1.108 (0.744-1.649)	.615	1.389 (0.835-2.309)	.205
Age, y				
≥50	—		—	
<50	1.194 (0.802-1.779)	.383	1.497 (0.900-2.490)	.121
Performance status				
0 or 1	—		—	
2	1.422 (1.023-1.977)	.036	1.528 (1.028-2.271)	.036
Trastuzumab therapy status				
First line	—		—	
≥Second line	1.365 (0.745-2.502)	.313	1.959 (0.962-3.986)	.064
No. of metastatic sites				
<3	—		—	
≥3	1.845 (1.166-2.920)	.009	2.378 (1.366-4.139)	.002

HR, hazard ratio;—, HR of 1.000.

proportion of breast cancers.[14-17,25] In a previous study, we identified intratumoral heterogeneity of *HER2* amplification in a subset of HER2-positive breast cancers and showed that *HER2* genetic and regional heterogeneity based on the *HER2*/CEP17 ratio was an independent predictor of poor prognosis in patients with primary HER2-postive breast cancer.[17] However, the importance of intratumoral *HER2* heterogeneity lies in not only its hindrance to accurate assessment of *HER2* status or its prognostic significance but also its possible association with treatment response to HER2-targeted therapy. In the present study, we showed that *HER2* regional heterogeneity based on the *HER2*/CEP17 ratio was a negative predictor of trastuzumab response in patients with HER2-positive metastatic breast cancer. Although *HER2* genetic heterogeneity by the *HER2*/CEP17 ratio was not a predictor of the response to trastuzumab, tumors in which a large proportion of cells had a *HER2*/CEP17 ratio of more than 2.2 or a HER2 IHC score of 3+ were clearly more responsive to trastuzumab. An obvious clinical implication of these findings is that patients with

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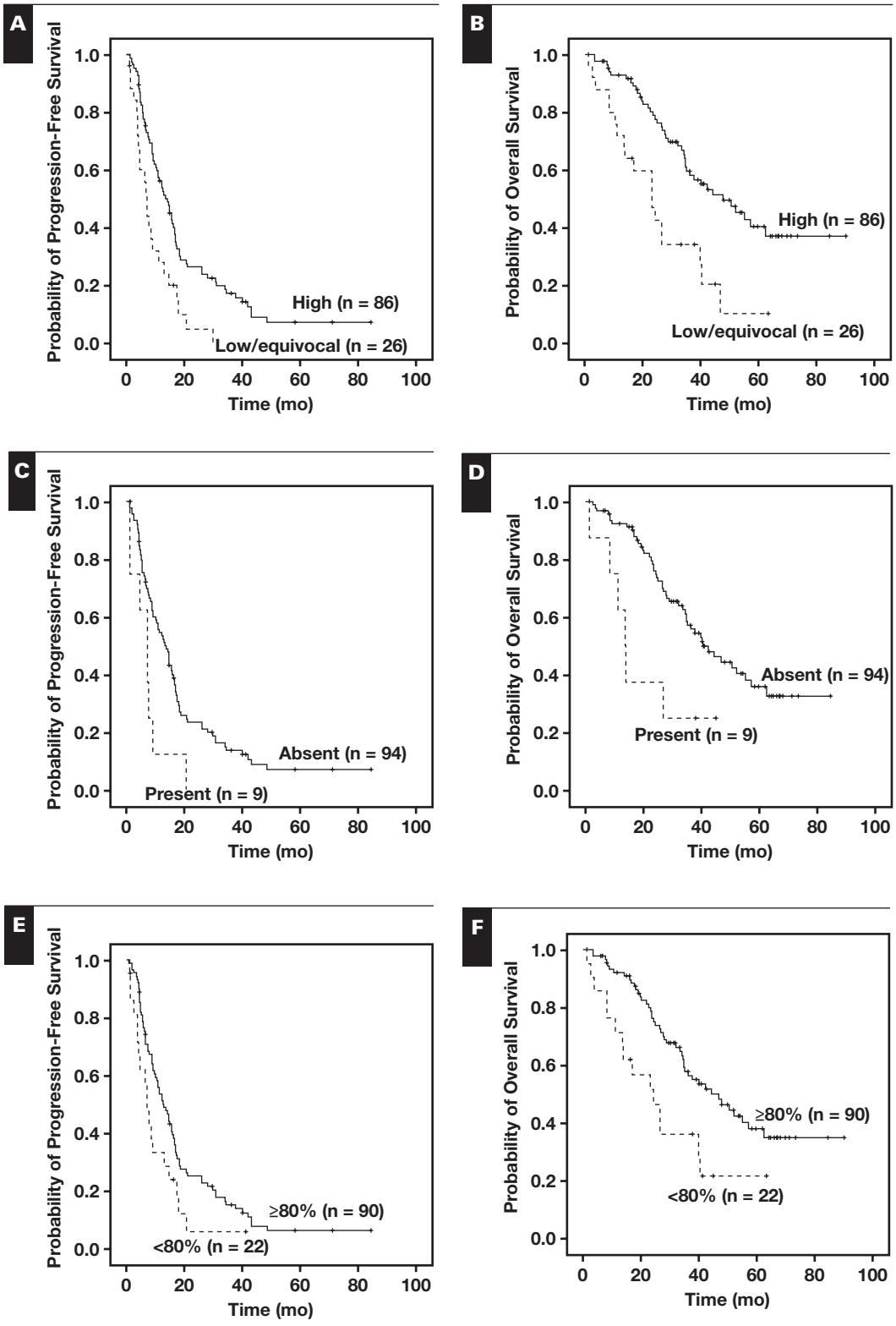

Figure 1 Progression-free and overall survival according to *HER2* status in patients with HER2-positive metastatic breast cancer treated with trastuzumab-based chemotherapy. Patients with tumors characterized by low-level or equivocal *HER2* amplification (**A**, **B**), *HER2* regional heterogeneity by *HER2*/CEP17 ratio (**C**, **D**), and a *HER2*/CEP17 ratio of more than 2.2 in less than 80% of tumor cells (**E**, **F**) had a significantly shorter progression-free and overall survival. **A**, $P = .002$. **B**, $P = .001$. **C**, $P = .015$. **D**, $P = .015$. **E**, $P = .033$. **F**, $P = .007$.

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Table 6
Impact of *HER2* Status on Time to Progression and Overall Survival in Multivariate Analyses

		Time to Progression		Overall Survival	
Model	**Variable**	**HR (95% CI)**	***P* Value**	**HR (95% CI)**	***P* Value**
A	Overall *HER2* gene amplification				
	High	—		—	
	Low or equivocal	2.284 (1.397-3.735)	.001	2.830 (1.606-4.986)	<.001
	Performance status				
	0 or 1	—		—	
	2	1.368 (0.976-1.918)	.069	1.585 (1.055-2.380)	.026
	No. of metastatic sites				
	<3	—		—	
	≥3	2.123 (1.327-3.396)	.002	2.915 (1.641-5.178)	<.001
B	*HER2* regional heterogeneity by *HER2*/CEP17 ratio				
	Absent	—		—	
	Present	2.475 (1.169-5.242)	.018	3.509 (1.461-8.426)	.005
	Performance status				
	0 or 1	—		—	
	2	1.534 (1.081-2.179)	.017	1.801 (1.162-2.790)	.008
	No. of metastatic sites				
	<3	—		—	
	≥3	2.063 (1.268-3.356)	.004	3.034 (1.676-5.492)	<.001
C	% of cells with a *HER2*/CEP17 ratio >2.2				
	≥80%	—		—	
	<80%	1.827 (1.091-3.058)	.022	2.498 (1.372-4.549)	.003
	Performance status				
	0 or 1	—		—	
	2	1.458 (1.046-2.035)	.026	1.716 (1.144-2.575)	.009
	No. of metastatic sites				
	<3	—		—	
	≥3	2.042 (1.279-3.258)	.003	2.777 (1.569-4.913)	<.001
D	% of cells with strong and complete membranous HER2 expression				
	≥75%	—			
	<75%	2.101 (1.233-3.580)	.006		
	Performance status				
	0 or 1	—			
	2	1.595 (1.137-2.237)	.007		
	No. of metastatic sites				
	<3	—			
	≥3	2.026 (1.259-3.260)	.004		

HR, hazard ratio;—, HR of 1.000.

heterogeneously amplified HER2-positive breast cancer are less likely to benefit from trastuzumab therapy. Instead, different treatment modalities may be necessary to target the HER2-negative subclones.

We also showed that patients with overall low-level or equivocal *HER2* amplification had significantly lower response rates to trastuzumab and shorter TTP and OS than did those with high-level amplification. Several previous studies, including ours, showed that low-level *HER2* amplification was associated with lower objective response rates to trastuzumab-based therapy and poorer clinical outcomes,[12,26,27] although they included small numbers of cases and used different cutoff values in scoring. Vogel et al[7] showed that there was little clinical benefit and no objective response to single-agent trastuzumab therapy in patients with HER2 2+ metastatic breast cancer. Similarly, Lipton et al[28] reported a reduced response to trastuzumab therapy in patients with metastatic *HER2* FISH-positive breast cancers with low-level HER2 expression compared with patients with high levels of HER2. Considering the close relationship between equivocal (2+) HER2 expression and low-level *HER2* gene amplification,[14] these results also support the positive correlation between the degree of *HER2* amplification and trastuzumab response. However, Gullo et al[29] reported findings to the contrary; in their study, patients with HER2-positive metastatic breast cancer with a high *HER2*/CEP17 ratio had shorter TTP and OS than did other patients when treated with a trastuzumab-based regimen. Furthermore, in the adjuvant setting, a predictive relationship between *HER2* FISH ratio and a differential benefit of trastuzumab was not found in early stage HER2-positive breast cancers.[30] Collectively, these findings indicate that further studies are warranted to confirm and elucidate the relationship between degree of *HER2* amplification and trastuzumab response.

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All cases with *HER2* genetic or regional heterogeneity based on the *HER2*/CEP17 ratio showed low-level or equivocal amplification of *HER2*. A HER2 protein expression score of 2+ or 1+ was more frequently observed in patients with *HER2* regional or genetic heterogeneity than in patients with homogeneous *HER2* status. This finding confirms the close association between intratumoral heterogeneity of *HER2* amplification and low-level *HER2* amplification or equivocal HER2 protein expression.[14-17] However, even in tumors with low-level amplification, the response rates to trastuzumab varied according to the presence of regional heterogeneity. In patients with tumors showing low-level *HER2* amplification and *HER2* regional heterogeneity by the *HER2*/CEP17 ratio, the clinical benefits and the objective response rates were lower than in patients with tumors showing homogeneous *HER2* status, although statistical significance was not reached (37.5% vs 55.6% for objective response; 50% vs 72.2% for clinical benefit).

Our findings raise an issue concerning the reporting of *HER2* in situ hybridization results in practice. It is evident from this study that *HER2* heterogeneity is clinically relevant to trastuzumab response. Thus, we propose that *HER2* in situ hybridization results should include information about *HER2* heterogeneity—for example, the proportion of tumor cells with a *HER2*/CEP17 ratio of more than 2.2, degree of overall *HER2*/CEP17 ratio, and regional heterogeneity (ie, the presence or absence of distinct regions of amplified cells). Moreover, since the proportion of tumor cells with a HER2 IHC score of 3+ was correlated with trastuzumab response, and heterogeneous HER2 expression could be a good indicator of *HER2* regional heterogeneity, it may be worthwhile to include the percentage of tumor cells with a score of 3+ in the reporting of HER2 IHC results. As suggested by UK guidelines[31] and our group,[17,32] if a tumor has differentially stained regions, these regions should all be considered in the assessment of gene status by *HER2* in situ hybridization. Scanning of the entire tumor section should be the first step in this process to identify any areas with different levels of amplification, and then at least three representative areas, including both amplified and nonamplified areas, should be selected to assess *HER2* regional heterogeneity. SISH may be superior, in this context, to FISH, which is why it was adopted in this study.

In a previous study, we identified *HER2* regional heterogeneity in 18% and genetic heterogeneity in 11% of 96 cases of primary HER2-positive breast cancer, using the *HER2*/CEP17 ratio.[17] In the present study, the proportion of tumors with *HER2* regional and genetic heterogeneity appears to be lower. A possible explanation is that *HER2* regional and genetic heterogeneity is closely associated with low-level or equivocal *HER2* amplification, and the frequency of low-level or equivocal *HER2* amplification was lower in the present (23%) than in the previous study (41%). Limitations of this study also include its retrospective nature, inhomogeneity of the samples (primary or metastatic, core biopsy, or excisional biopsy), and the different therapeutic regimens used across the study population. Further large-scale prospective studies may be warranted to confirm the clinical significance of *HER2* heterogeneity to treatment outcomes of HER2-targeted therapy.

In summary, *HER2* regional heterogeneity was identified in 8.7% and genetic heterogeneity in 2.7% of cases of HER2-positive metastatic breast cancer, based on the *HER2*/CEP17 ratio. *HER2* regional heterogeneity by the *HER2*/CEP17 ratio, the overall level of *HER2* gene amplification, and the proportion of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 or a HER2 protein expression score of 3+ were independent predictors of response to trastuzumab-containing therapy. We conclude that a detailed assessment of *HER2* status, including *HER2* amplification levels, *HER2* regional heterogeneity, and the percentage of tumor cells with a *HER2*/CEP17 ratio of more than 2.2 or a HER2 protein expression score of 3+, can provide valuable information about the predicted response to trastuzumab-based therapy in patients with HER2-positive metastatic breast cancer.

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