CME/SAM

Clinicopathologic Significance of the Intratumoral Heterogeneity of *HER2* Gene Amplification in HER2-Positive Breast Cancer Patients Treated With Adjuvant Trastuzumab

Hee Jin Lee, MD, PhD,[1] Joo Young Kim, MD,[2] So Yeon Park, MD, PhD,[3,4] In Ah Park, MD,[1] In Hye Song, MD,[1] Jong Han Yu, MD, PhD,[5] Jin-Hee Ahn, MD, PhD,[6] and Gyungyub Gong, MD, PhD[1]

From the [1]Department of Pathology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea; [2]Department of Pathology, Korea University Anam Hospital, Korea University College of Medicine, Seoul, Korea; [3]Department of Pathology, Seoul National University Bundang Hospital, Seongnam, Gyeonggi, Korea; [4]Department of Pathology, Seoul National University College of Medicine, Seoul, Korea; and Departments of [5]Surgery and [6]Oncology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea.

Key Words: Breast carcinoma; Gene amplification; HER2; Heterogeneity; Trastuzumab; Adjuvant chemotherapy

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ABSTRACT

Objectives: *Although intratumoral heterogeneity of human epidermal growth factor receptor 2 (*HER2*) gene amplification has been associated with a poor prognosis for primary HER2-positive breast cancer and metastatic HER2-positive breast cancer treated with trastuzumab, the clinicopathologic significance in a setting involving trastuzumab treatment as an adjuvant treatment has not been studied in patients.*

Methods: *We retrospectively investigated 443 patients with HER2-positive breast cancer treated with surgery, adjuvant chemotherapy, and 1 year of trastuzumab. Three areas that showed different levels of HER2 protein expression were chosen, and silver in situ hybridization was performed.*

Results: HER2 *regional and genetic heterogeneity was found in 6.2% and 6.8% of tumors, respectively. Both types of heterogeneity were significantly associated with hormone receptor positivity, HER2 immunohistochemistry score of 2+, a low level of* HER2 *gene amplification, and absence of an extensive intraductal component. Genetic heterogeneity also showed strong correlation with a lower histologic grade. In the hormone receptor–positive group, the regional heterogeneity affected disease-free survival of patients (hazard ratio, 4.869; 95% confidence interval, 1.424-16.646;* P = *.005), whereas genetic heterogeneity did not.*

Conclusions: *Evaluation of intratumoral heterogeneity, especially in cases with hormone receptor positivity, may be valuable for assessing the prognosis of HER2-positive patients anticipating treatment with adjuvant systemic therapy and trastuzumab.*

Upon completion of this activity you will be able to:
- provide a working definition of *HER2* gene amplification and genetic heterogeneity.
- discuss the clinical significance of HER2 genetic heterogeneity.

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An accurate assessment of human epidermal growth factor receptor 2 (*HER2*) status in breast cancer is essential since agents targeting HER2 have a proven effectiveness for patients with HER2-positive breast cancer, which is associated with an aggressive phenotype and poor prognosis.[1-3] Trastuzumab, a humanized monoclonal antibody that recognizes the extracellular portion of the HER2 protein, has been demonstrated to be effective in patients with HER2-positive metastatic breast cancer and in patients with operable HER2-positive breast cancer as an adjuvant treatment.[4-9] However, similar to other targeted agents, primary and secondary resistance to trastuzumab has been frequently reported.[4-9]

Previously, we showed that *HER2* regional heterogeneity, which is defined as the existence of amplification-negative or amplification-equivocal patterns in different areas of the tumor, in which the overall level of *HER2* gene amplification and the percentage of tumor cells has a *HER2*/CEP17 ratio of more than 2.2 or a HER2 protein expression score of 3+, is an independent predictor of response to trastuzumab-containing therapy in patients with

HER2-positive metastatic breast cancer.[10] In other studies, patients with metastatic breast cancer with a 2+ HER2 immunohistochemistry (IHC) score are less responsive to trastuzumab than those with a 3+ score.[5,7] Low-level *HER2* amplification is associated with decreased time to progression compared with high-level amplification in patients with metastatic breast cancer treated with weekly paclitaxel and trastuzumab.[11] In addition, low-level *HER2* amplification correlates with a lower rate of pathologic response to neoadjuvant trastuzumab-based therapy.[12] However, HER2 staining intensity and the level of *HER2* gene amplification are not associated with trastuzumab benefit in patients with HER2-positive breast cancer treated with adjuvant chemotherapy and 1 year of trastuzumab.[13,14] Pogue-Geile et al,[15] after analyzing the gene expression profiles of 462 genes with an nCounter assay (NanoString Technologies, Seattle, WA), identified a subset of patients who received no benefit from trastuzumab, whose tumors were characterized by intermediate-level *HER2* and high level of *ESR1* (estrogen receptor α) messenger RNA expression. Even though patients with a low level of *HER2* gene amplification could benefit from trastuzumab as an adjuvant, and adjuvant trastuzumab is a standard therapeutic modality for the treatment of HER2-positive breast cancer regardless of *HER2* gene amplification level, prognostic factors of this patient subgroup are still unclear.

HER2 heterogeneity has been found in a subset of breast cancers, with a wide range of reported incidence rates (1.3%-54%).[16-23] *HER2* heterogeneity has been linked to a low level of *HER2* amplification or an equivocal HER2 expression.[20,23-25] *HER2* heterogeneity may contribute to inaccurate assessment of *HER2* status and affect treatment decisions, and this variability could be associated with a reduced response to HER2-targeted therapy. However, the prognostic significance of *HER2* intratumoral heterogeneity has not been studied extensively in an adjuvant setting. Hence, to determine the clinical significance of *HER2* heterogeneity and low-level amplification on trastuzumab treatment in HER2-positive breast cancer, we analyzed 443 cases of HER2-positive breast cancer treated with adjuvant chemotherapy and 1 year of trastuzumab. We also sought to identify the clinicopathologic characteristics of cases with *HER2* heterogeneity and low-level amplification.

Materials and Methods

Patients and Tissue Specimens

A total of 443 patients with HER2-positive breast cancer who underwent surgery for primary breast cancer between 2005 and 2011 at the Asan Medical Center, who had available formalin-fixed, paraffin-embedded tissue samples with successful *HER2* silver in situ hybridization (SISH) results for analysis, were included in this study as previously described.[26] All patients were preoperatively chemotherapy and radiotherapy naive, and all underwent adjuvant treatment. Of 443 patients, 161 with node-negative breast cancer were treated with four cycles of adjuvant anthracycline and cyclophosphamide and 1 year of trastuzumab. The remaining 282 patients had node-positive breast cancer and were treated with four cycles of anthracycline and cyclophosphamide, followed by four cycles of paclitaxel or docetaxel and 1 year of trastuzumab. Trastuzumab was given every 3 weeks. Clinicopathologic information was obtained from the patients' medical records and surgical pathology reports.

Expressions of standard biomarkers, including estrogen receptor (ER), progesterone receptor (PR), and HER2, were reviewed in full sections that were immunohistochemically stained at the time of diagnosis. ER and PR levels were regarded as positive if there was at least 1% positive tumor nuclei staining.[27] The hormone receptor–positive group was defined as either ER- or PR-positive tumors. HER2-overexpressing tumors were defined as those with scores of 3+ by IHC or gene amplification by fluorescence in situ hybridization or SISH.[28] Exemption from informed consent after deidentification of information was approved by the Asan Medical Center Institutional Review Board.

Tissue Microarray Construction

Formalin-fixed, paraffin-embedded tissue samples were arrayed with a tissue-arraying instrument. After review of a whole slide of tumor section immunostained with anti-HER2, three areas were selected and arrayed in 1-mm diameter cores. If the tumor showed a heterogeneous staining pattern for HER2 IHC, differentially stained areas were chosen.

SISH Assays for *HER2* Gene Amplification

Automated SISH assays on tissue microarray sections were performed with INFORM HER2 DNA and Chromosome 17 probes (Ventana Medical Systems, Oro Valley, AZ) using an ultraView SISH Detection Kit (Ventana Medical Systems) according to the manufacturer's protocols.[29] Both probes are prelabeled with dinitrophenol (DNP) and are optimally formulated for use with the ultraView SISH Detection Kit and accessory reagents from Ventana's BenchMark series of automated slide stainers. The *HER2* DNA probe was denatured at 95°C for 12 minutes and hybridized at 52°C for 6 hours, followed by three washes of the appropriate stringency at 72°C. The chromosome 17 (CEP17) probe was denatured at 95°C for 12 minutes and hybridized at 44°C for 6 hours on the same slide, followed by three additional washes at 72°C at the

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appropriate stringency. The probes were visualized using a rabbit anti-DNP primary antibody and the ultraView SISH Detection Kit, which contains goat anti–rabbit secondary antibody conjugated to horseradish peroxidase as the chromogenic enzyme. After sequential addition of silver acetate, hydroquinone, and H_2O_2, the silver precipitate was deposited in the nuclei, producing a black signal for each copy of the *HER2* gene. A red dot representing CEP17 appeared following a reaction with Fast Red and naphthol phosphate. The specimen was counterstained with Harris hematoxylin.

We assessed the *HER2* gene amplification status in the three tissue microarrays. Fifty cells were evaluated in each core, where possible a total of 150 cells were counted for each case. The genetic variables reported included *HER2* gene copy number, CEP17 copy number, and the ratio of *HER2* gene copies to that of CEP17. 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 2013 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) criteria.[28] The *HER2* gene was considered amplified if the average ratio of *HER2* to CEP17 signals was 2.0 or more or a ratio less than 2.0 and the average HER2 copy number was 6 or more. A ratio of 4.0 or higher was considered high-level amplification; a ratio 2.0 or higher and less than 4.0 was considered low-level amplification. Cases with a ratio less than 2.0 and average *HER2* copy numbers of 4 or more and less than 6 were considered equivocal for amplification. The cutoff values for CEP17 copy number changes were adopted from Ma et al[30] 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 CEP17 (ie, 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.[20] 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 with modification, as the existence of tumor cells with a *HER2*/CEP17 ratio of 2 or more in 5% to 50% of all examined tumor cells.[31]

Histologic Evaluation

H&E-stained sections were histopathologically analyzed as previously described.[26]

Assessment of Trastuzumab Response and Statistical Analysis

Disease-free survival (DFS) was defined as the time from the start of trastuzumab to recurrence of breast cancer at any site. 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. A receiver operating characteristic (ROC) curve analysis was performed to identify the most appropriate cutoff values for the percentages of cells with a *HER2*/CEP17 ratio of 2 or more and with a HER2 IHC of 3+. 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 (HRs) and their 95% confidence intervals (CIs) were estimated for all factors. The threshold for statistical significance was set at 5% ($P < .05$), and all statistical analyses were performed with SPSS statistical software (version 18; SPSS, Chicago, IL). All P values are two-tailed.

Results

Clinicopathologic Characteristics of the Study Population

All 443 patients enrolled were women, and their median age at diagnosis was 49 years (range, 22-79 years). Tumor sizes ranged from 0.1 to 8.2 cm (median, 2.2 cm). Of the cases, 206 were pT1 tumors, 222 were pT2, and 15 were pT3. In lymph node analysis, 161 tumors were pN0 stage, 14 were pN1mi, 160 were pN1, 60 were pN2, and 48 were pN3. The median follow-up was 55.5 months (range, 25.4-112.3 months).

HER2 Amplification and Intratumoral Heterogeneity

HER2 SISH was successfully performed in all 443 cases of HER2-positive breast cancer. However, eight cases had only one assessable core, while 29 cases had two cores, and 406 cases had three cores. Therefore, regional heterogeneity could be assessed in 435 cases. In total, 345 (77.9%) cases showed high-level amplification (*HER2*/CEP17 ratio, 7.7 ± 2.5), and 98 (22.1%) cases showed low-level amplification (2.9 ± 0.7). Of the 345 high-level amplification cases, 310 (89.9%) cases had three assessable cores, 29 (8.4%) cases had two cores, and six (1.7%) cases had one core. Regional heterogeneity was identified in one (0.3%) case among 339 assessable high-level amplification cases. Of the 98 low-level amplification cases, 96 (98.0%) cases had three assessable cores, and two (2.0%) cases had one assessable core. Regional heterogeneity was identified in 26 (27.1%) of 96 assessable low-level amplification cases. Thus, of the 435 cases with two or three assessable cores, 27 (6.2%) cases had

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Table 1
Intratumoral Heterogeneity of *HER2* Gene Amplification

Pattern of *HER2* Regional Heterogeneity	*HER2*/CEP17 Ratio				HER2 IHC	% of Cells With HER2 3+	*HER2* Genetic Heterogeneity	% of Cells With *HER2*/CEP17 Ratio ≥2.2	Average CEP17
	Area 1	Area 2	Area 3	Overall					
High-level amplification/negative (n = 4, 0.9%)	4.02	0.98	1.30	2.27	2	5	1	36.0	1.8
	9.64	1.49	4.12	4.81	3	40	0	58.7	1.4
	1.09	8.40	1.00	3.74	3	30	1	33.3	1.5
	6.40	1.25	1.53	2.72	2	5	1	38.0	2.0
High-level amplification/low-level amplification/equivocal (n = 1, 0.2%)	4.5	2.33	1.91	2.97	3	30	0	58.7	2.5
Low-level amplification/negative (n = 19, 4.4%)	2.75	2.57	1.02	2.07	3	30	0	59.0	2.1
	2.73	1.25	1.31	1.62	2	0	1	30.0	1.5
	1.94	2.55	2.50	2.34	2	0	1	48.0	1.5
	1.47	1.88	2.13	1.83	2	5	1	25.3	2.0
	1.85	1.81	2.75	2.06	2	0	1	42.7	1.7
	2.77	1.58	1.74	1.99	1	0	1	33.3	1.3
	1.53	3.11	1.71	2.15	2	5	1	27.3	1.7
	1.91	1.86	2.14	1.96	2	0	1	40.0	1.9
	1.63	2.45	2.64	2.21	2	0	1	47.3	1.9
	1.63	2.09	1.49	1.73	2	0	1	24.0	1.8
	2.06	2.33	1.86	2.08	2	0	1	45.3	1.8
	1.77	2.24	2.00	2.00	2	0	1	38.7	1.5
	2.25	1.78	1.62	1.89	2	5	1	32.0	1.7
	2.58	2.10	1.95	2.19	3	80	1	40.7	1.8
	2.15	1.74	1.65	1.85	2	0	1	28.0	1.6
	3.36	1.93	2.44	2.55	2	0	1	46.7	1.6
	2.04	1.70	1.92	1.88	2	0	1	32.0	1.6
	2.1	1.75	2.38	2.08	2	5	1	40.7	1.6
	1.7	2.14	1.87	1.90	2	0	1	40.0	2.0
Low-level amplification/equivocal (n = 3, 0.7%)	1.89	1.80	2.02	1.90	2	0	1	34.7	2.7
	2.15	1.85	1.58	1.84	2	5	1	28.0	2.6
	1.94	1.98	2.45	2.09	2	5	0	52.0	3.0

CEP17, chromosome 17; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

regional heterogeneity of *HER2* based on the *HER2*/CEP17 ratio **Table 1**. Among the 27 cases, four (0.9%) showed high-level/negative amplification, one (0.2%) case showed a mixture of high-level/low-level/equivocal amplification, 19 (4.4%) cases showed mixed low-level/negative amplification, and three (0.7%) cases showed low-level/equivocal amplification.

The genetic heterogeneity of *HER2* was identified in 30 (6.8%) cases; one case was with high-level amplification, and 29 cases were with low-level amplification. Of these cases, 23 showed regional heterogeneity, six did not, and one could not be assessed (Table 1). The *HER2* regional and genetic heterogeneity was significantly correlated ($P < .001$).

Association of *HER2* Heterogeneity and Clinicopathologic Characteristics

The relationship between *HER2* heterogeneity and various clinicopathologic characteristics is summarized in **Table 2**. The *HER2* regional heterogeneity was correlated with the absence of an extensive intraductal component (EIC), higher pT stage, and hormone receptor positivity ($P = .001$, $P = .022$, and $P < .001$, respectively). *HER2* genetic heterogeneity was more frequently found in the cases with lower histologic grade, absence of EIC, and hormone receptor positivity ($P = .005$, $P = .003$, and $P = .001$, respectively). Also, cases with genetic and regional heterogeneity displayed a greater association with 2+ HER2 protein expression than 3+ expression, as well as low-level rather than high-level *HER2* amplification ($P < .001$). The *HER2*/CEP17 ratio was significantly lower in cases with *HER2* heterogeneity than those without (2.3 ± 0.7 vs 6.9 ± 2.8, $P < .001$ in terms of regional heterogeneity and 2.3 ± 1.1 vs 7.0 ± 2.8, $P < .001$ in terms of genetic heterogeneity). Low-level amplification of *HER2* was associated with lower histologic grade ($P = .013$), absence of EIC, hormone receptor positivity, 2+ HER2 IHC score, and the presence of polysomy ($P < .001$).

HER2 Status and Clinical Outcomes

Because a previous study of metastatic HER2-positive breast cancer cases involving trastuzumab treatment asserted a predictive value to evaluating the percentage of tumor cells with a HER2 IHC score of 3+ and percentage of tumor cells with a *HER2*/CEP17 ratio of more than 2.2,[10] we also

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Table 2
Clinicopathologic Characteristics in Relation to *HER2* Regional and Genetic Heterogeneity and *HER2* Amplification Level

	Regional Heterogeneity			Genetic Heterogeneity		
Characteristic	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**
Histologic grade						
2	148 (36.3)	14 (51.9)	.149	148 (35.8)	19 (63.3)	.005
3	260 (63.7)	13 (48.1)		265 (64.2)	11 (36.7)	
Extensive intraductal component						
Absent	247 (60.5)	25 (92.6)	.001	249 (60.3)	26 (86.7)	.003
Present	161 (39.5)	2 (7.4)		164 (39.7)	4 (13.3)	
pT						
1	194 (47.5)	7 (25.9)	.022	194 (47.0)	12 (40.0)	.322
2	201 (49.3)	18 (66.7)		206 (49.9)	16 (53.3)	
3	13 (3.2)	2 (7.4)		13 (3.1)	2 (6.7)	
Lymph node metastasis						
Absent	149 (36.5)	9 (33.3)	.838	150 (36.3)	11 (36.7)	1
Present	259 (63.5)	17 (66.7)		263 (63.7)	19 (63.3)	
Lymphovascular invasion						
Absent	235 (57.9)	16 (59.3)	1	239 (58.2)	18 (60.0)	1
Present	171 (42.1)	11 (40.7)		172 (41.8)	12 (40.0)	
Hormone receptor expression						
Negative	229 (56.1)	5 (18.5)	<.001	231 (55.9)	7 (23.3)	.001
Positive	179 (43.9)	22 (81.5)		182 (44.1)	23 (76.7)	
HER2 immunohistochemistry						
2	37 (9.1)	22 (81.5)	<.001	35 (8.5)	27 (90.0)	<.001
3	371 (90.9)	5 (18.5)		378 (91.5)	3 (10.0)	
Polysomy						
Absent	356 (87.3)	23 (85.2)	.766	359 (86.9)	27 (90.0)	.783
Present	52 (12.7)	4 (14.8)		54 (13.1)	3 (10.0)	
HER2 gene amplification level						
Low-level amplification	70 (17.2)	26 (96.3)	<.001	69 (16.7)	29 (96.7)	<.001
High-level amplification	338 (82.8)	1 (3.7)		344 (83.3)	1 (3.3)	
HER2/CEP17 ratio	6.9 ± 2.8	2.3 ± 0.7	<.001	7.0 ± 2.8	2.3 ± 1.1	<.001

CEP17, chromosome 17; HER2, human epidermal growth factor receptor 2.
[a] Values are presented as number (%) unless otherwise indicated.

assessed the prognostic significance of those variables. ROC curve analysis identified cutoff points of 65% for the percentage of tumor cells with a HER2 IHC score of 3+ and 75% for the percentage of tumor cells with a *HER2*/CEP17 ratio of 2 or more.

We investigated whether the clinical outcomes in our study patients were associated with various clinicopathologic parameters, including the *HER2* status. In univariate analysis, patients with regional heterogeneity had a significantly shorter DFS than those without (HR, 2.828; 95% CI, 1.092-7.327; $P = .025$). A higher pT stage (HR, 1.004; 95% CI, 1.000-1.007; $P = .026$) and the presence of lymphovascular invasion (HR, 2.85; 95% CI, 1.382-5.879; $P = .005$) were also associated with a short DFS. In our hormone receptor–negative cases, a higher pT stage was significantly associated with a poor DFS **Table 3**. In our hormone receptor–positive cases, the presence of lymphovascular invasion and *HER2* regional heterogeneity were associated with a shorter DFS ($P = .022$ and .005, respectively) **Figure 1**. Also, a greater percentage of cells with a *HER2*/CEP17 ratio of 2 or more was associated with a shorter DFS in the hormone receptor–positive cases (<75% vs ≥75%; HR, 3.766; 95% CI, 1.149-12.340; $P = .019$) **Figure 2**. Other factors, including lymph node metastasis, EIC, HER2 IHC score, *HER2* SISH amplification status, *HER2* genetic heterogeneity, and the percentage of tumor cells with a HER2 expression score of 3+, were not correlated with the DFS.

In multivariate analyses including pT stage, lymphovascular invasion, and *HER2* regional heterogeneity, only the presence of lymphovascular invasion was an independent negative prognostic factor (HR, 2.673; 95% CI, 1.280-5.582; $P = .009$) in all cases. In the hormone receptor–positive cases, the presence of regional heterogeneity (HR, 4.993; 95% CI, 1.459-17.088; $P = .010$) and the percentage of cells with a *HER2*/CEP17 ratio of 2 or more (<75% vs ≥75%; HR, 4.570; 95% CI, 1.388-16.045; $P = .012$) were observed to be independent prognostic factors, as was the presence of lymphovascular invasion (multivariate analysis with regional heterogeneity: HR, 0.614; 95% CI, 1.319-28.330; $P = .021$; multivariate analysis with the percentage of cells with a *HER2*/CEP17 ratio ≥2: HR, 7.023; 95% CI, 1.507-32.721; $P = .013$).

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Table 3
Univariate Analyses of Disease-Free Survival

Variable	Hormone Receptor Negative		Hormone Receptor Positive	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
pT stage: T2-T3 vs T1	2.845 (1.113-7.271)	.029	1.937 (0.514-7.301)	.329
Lymph node metastasis: positive vs negative	1.901 (0.699-5.169)	.208	5.245 (0.666-41.283)	.115
Histologic grade: 3 vs 2	1.279 (0.472-3.467)	.629	1.279 (0.390-4.191)	.685
Lymphovascular invasion: positive vs negative	2.194 (0.937-5.133)	.070	5.987 (1.293-27.724)	.022
Extensive intraductal component: positive vs negative	1.743 (0.753-4.035)	.195	1.134 (0.332-3.874)	.841
HER2 immunohistochemistry: 1+ or 2+ vs 3+	1.776 (0.525-6.005)	.356	1.390 (0.369-5.240)	.627
Percentage of cells with HER2 3+ expression: <65% vs ≥65%	1.648 (0.557-4.871)	.362	2.190 (0.667-7.189)	.185
HER2 SISH status: low-level vs high-level	1.008 (0.548-1.853)	.980	0.732 (0.404-1.326)	.296
Percentage of cells with a *HER2*/CEP17 ratio ≥2: <75% vs ≥75%	1.387 (0.410-4.694)	.598	3.766 (1.149-12.340)	.019
HER2 regional heterogeneity: positive vs negative	2.243 (0.301-16.694)	.418	4.869 (1.424-16.646)	.005
HER2 genetic heterogeneity: positive vs negative	0.048 (0-3231.248)	.419	2.989 (0.793-11.269)	.089

CEP17, chromosome 17; CI, confidence interval; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; SISH, silver in situ hybridization.

Discussion

The importance of intratumoral heterogeneity of *HER2* amplification has been recently recognized.[10,20,23-25,32] Previously, we showed that *HER2* genetic heterogeneity is more commonly observed in hormone receptor–positive, HER2-positive cancers than in hormone receptor–negative cancers.[26] *HER2* heterogeneity may contribute to an inaccurate assessment of the *HER2* status and affect treatment decisions. Thus, the evaluation of *HER2* heterogeneity may be clinically important. However, studies of the relationship between *HER2* heterogeneity and treatment effects or clinical outcomes are limited, and the prognostic significance of *HER2* intratumoral heterogeneity has not been extensively studied in an adjuvant setting. In our current study, we identified the prognostic significance of *HER2* heterogeneity in a large number of HER2-positive breast cancer patients treated with adjuvant trastuzumab.

In a previous study, the intratumoral heterogeneity of *HER2* amplification was associated with a shorter DFS in primary HER2-positive breast cancer; however, only a small number of patients received adjuvant trastuzumab in that report.[20] In HER2-positive metastatic breast cancer with trastuzumab-based chemotherapy, a low-level *HER2* amplification and *HER2* regional heterogeneity have been associated with shorter time to progression and lower overall survival.[10] In our current study, we find that most cases with *HER2* regional or genetic heterogeneity involve hormone receptor–positive tumors. In our hormone receptor–positive group, *HER2* regional heterogeneity was an independent indicator of a poor DFS in HER2-positive breast cancer. Since the presence of lymphovascular invasion was the only prognostic factor evident in this group, we surmise that the evaluation of intratumoral heterogeneity may be important for assessing the prognosis, especially in hormone receptor– and HER2-positive breast cancer treated with adjuvant trastuzumab.

Besides hormone receptor positivity, *HER2* heterogeneity was correlated with the absence of EIC, a 2+ HER2 protein expression score, and a low level of *HER2* amplification. These were features previously associated with hormone receptor positivity in this study group.[26] Our current results are consistent with previous reports showing that *HER2* heterogeneity correlated with a low level of amplification and equivocal HER2 IHC score.[20,23-25]

The incidence of *HER2* heterogeneity is variable, ranging from 1.3% to 54%.[10,16-23] This variable range may be due to diverse characteristics of the study groups, such as differing ethnic backgrounds and evaluating methods for *HER2* gene amplification. In our current study, the rates of *HER2* genetic and regional heterogeneity were measured at 6.8% and 6.2%, respectively. The mechanism of intratumoral heterogeneity has not been clearly demonstrated in our current analyses, but our present data do indicate that the addition of *HER2* amplification might be beneficial for genetically unstable originally *HER2*-negative tumors.[20,33,34]

Previously, we found that low-level amplification is more frequent in hormone receptor–positive, HER2-positive cancers than in hormone receptor–negative cases ($P < .001$).[26] Even though this difference was statistically significant ($P < .001$), the absolute difference in average *HER2*/CEP17 ratio was not large between the hormone receptor–negative and hormone receptor–positive groups (7.2 ± 2.8 for the hormone receptor–negative group; 6.0 ± 3.0 for the hormone receptor–positive group). In contrast, the average *HER2*/CEP17 ratio was only 2.3 for cases with genetic and regional heterogeneity. Of 27 cases with regional heterogeneity in our current study, 24 cases showed low-level/negative or low-level/equivocal amplification. Hence, tumors with intratumoral heterogeneity of *HER2* amplification are predominantly those that have the lowest level of *HER2* gene amplification. Because the 2013 updated ASCO/CAP guideline recommendations for HER2 testing include less

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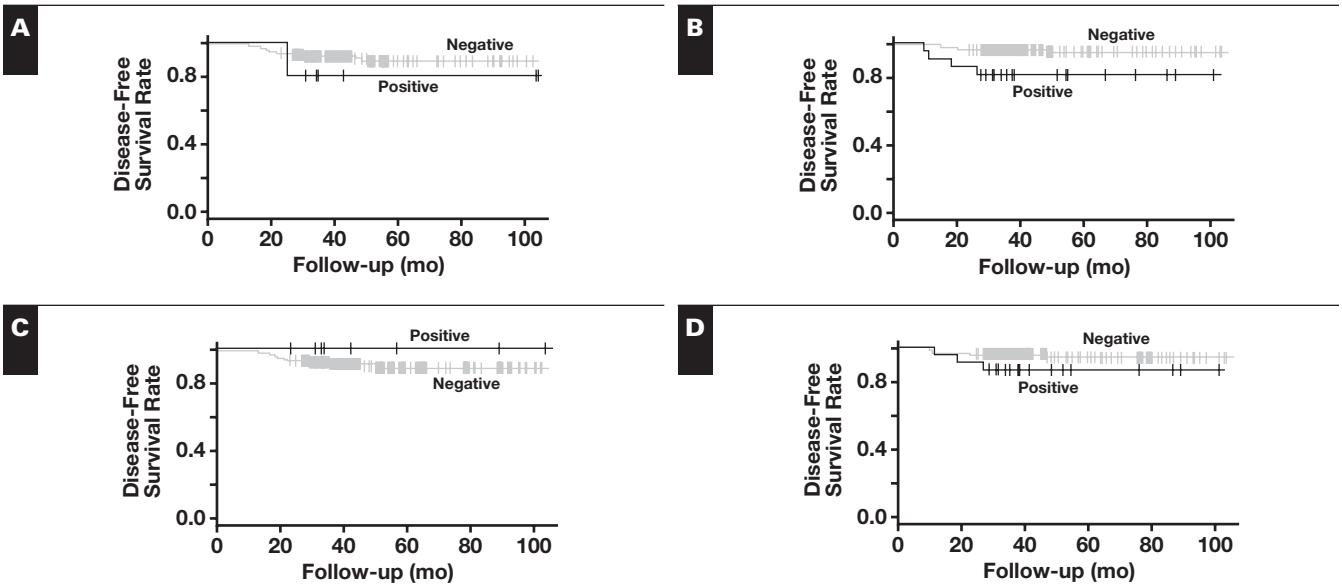

Figure 1 Measurement of disease-free survival comparing the effect of *HER2* regional (**A** and **B**) and genetic (**C** and **D**) heterogeneity in hormone receptor–negative (**A** and **C**) and hormone receptor–positive (**B** and **D**) subgroups. Cases with *HER2* regional heterogeneity show a decreased disease-free survival rate vs those without heterogeneity in the hormone receptor–positive subgroup. *HER2* genetic heterogeneity was not significantly associated with disease-free survival.

Figure 2 Analysis of the percentages of tumor cells with a *HER2*/CEP17 ratio of 2 or more on disease-free survival in hormone receptor–negative (**A**) and hormone receptor–positive (**B**) subgroups. The hormone receptor–positive subgroup cases in which the percentage of tumor cells with a *HER2*/CEP17 ratio of 2 or more is 75% or less show a decreased disease-free survival rate vs those tumors where that percentage is 75% or higher.

strict criteria for an IHC score of 2+ (more than 10% of tumor cells presenting weak/moderate incomplete membrane staining) than the 2007 guideline (more than 10% of tumor cells presenting weak/moderate complete membrane staining or within 10% of tumor cells presenting complete and circumferential membrane staining that is intense), equivocal test results for IHC, which need an intrasample confirmatory test (same specimen using in situ hybridization) or an intersample test (new specimen using IHC or in situ hybridization), might be increasing.[28,35,36] Thus, cases with the lowest level of *HER2* gene amplification and heterogeneity would be increasing under the 2013 standard compared with the 2007 standard.

HER2 staining intensity and the level of *HER2* gene amplification are not associated with trastuzumab benefit in patients with HER2-positive breast cancer treated with adjuvant chemotherapy and 1 year of trastuzumab compared with patients without the adjuvant trastuzumab.[13,14] However, the clinicopathologic significance of low-level *HER2* gene amplification has not been studied in patients with breast cancer receiving trastuzumab as an adjuvant. In our survival analysis, after dividing tumors into low-level and high-level *HER2* gene amplification, we could not find a difference in DFS rates. However, using ROC curve analysis, we found a cutoff point of 75% where the percentage of cells with a *HER2*/CEP17 ratio of 2 or more was significantly associated, dividing hormone receptor–positive patients into two groups with different DFS rates. Therefore, additional treatment modalities might be appropriate for tumors possessing *HER2* regional heterogeneity or a lower percentage of cells with a *HER2*/CEP17 ratio of 2 or more.

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Current observations appear to indicate that *HER2* heterogeneity is clinically relevant to the response of adjuvant trastuzumab treatment. Thus, we recommend as a routine practice when reporting *HER2* in situ hybridization to report information about *HER2* heterogeneity, including regional heterogeneity or the percentage of amplified cells. Since HER2 protein expression level is correlated with *HER2* gene amplification level, screening of the whole slide section by HER2 IHC might also be helpful.[13]

In summary, *HER2* regional and genetic heterogeneity was found in 6.2% and 6.8%, respectively, of HER2-positive primary breast cancers with adjuvant trastuzumab treatment. Both types of heterogeneity were significantly associated with hormone receptor positivity, HER2 IHC 2+, a low level of *HER2* gene amplification, and the absence of EIC. In the hormone receptor–positive group, regional heterogeneity of *HER2* gene amplification affected the DFS of patients and was an independent negative prognostic indicator. We recommend the evaluation of intratumoral heterogeneity, especially in cases with hormone receptor positivity, to assess the prognosis of HER2-positive patients being treated with systemic therapy and trastuzumab as an adjuvant.

Corresponding author: Gyungyub Gong, MD, PhD, Dept of Pathology, University of Ulsan College of Medicine, Asan Medical Center, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Korea; gygong@amc.seoul.kr.

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