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HER2 Equivocal Status in Early Breast Cancer Is Not Associated with Higher Risk of Recurrence

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Abstract. *Aim: The primary aim of the study was to assess the association between risk of recurrence and HER2 equivocal gene status through immunohistochemistry in patients with early breast cancer. Patients and Methods: We retrospectively analyzed clinical and pathological data of 455 consecutive patients with early breast cancer (BC) who were HER2$^+$ and had a HER2/CEP17 ratio <2.0, who underwent surgery at the European Institute of Oncology after 2007. The role of HER2/CEP17 ratio on recurrence-free survival was assessed with univariate and multivariate Cox regression models. Results: We found no significant association between risk of recurrence and HER2 equivocal testing in patients with early breast cancer. In subgroup analysis, a significant interaction between HER2/CEP17 ratio and nodal involvement was observed (p=0.02). Conclusion: Patients with HER2 equivocal status have no significantly higher risk of recurrence.*

Overexpression of the human epidermal growth factor receptor type 2 (HER2) occurs in approximately 15% to 20% of invasive breast cancers and is associated with poor clinical outcome (1-3). Trastuzumab, a humanized monoclonal antibody that binds HER2, improves the outcomes for patients with HER2-positive breast cancer (BC). Four phase 3 randomized trials involving more than 8,000 patients showed that trastuzumab decreased the risk of recurrence by approximately 50% and improved overall survival (4-7). By applying immunohistochemistry (IHC) to assess protein overexpression, BC can be classified into two major groups: HER2-positive (score 3+) BC and HER2-negative (score 0 and 1+) BC. However, up to 18% of all newly-diagnosed BC have an equivocal HER2 expression (score 2+) (8). In this group, HER2 positivity must be confirmed by fluorescence *in situ* hybridization (FISH). BC often shows heterogeneity in HER2 overexpression and amplification, with only a fraction of tumor cells being HER2-positive. Thus far, only patients with HER2 positivity, defined as either 3+ by IHC or FISH amplified defined as a *HER2* gene to chromosome 17 (HER2/CEP17) ratio ≥2.0, are suitable to receive trastuzumab treatment. Limited data are available on clinico-pathological significance of HER2 equivocal status and on the prognostic role HER2 2+ and FISH test with a HER2/CEP17 ratio <2.0 in patients with early breast cancer. We designed the present study to provide a definitive portrait of this controversial category of breast cancers. The primary aim of the study was to assess the prognostic role of equivocal IHC HER2 status with a HER2/CEP17 ratio <2.0 in patients with early breast cancer.

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Key Words: Breast cancer, equivocal HER2 status, HER2 amplification, heterogeneity.

Materials and Methods

We retrospectively analyzed data from 455 consecutive early BC patients with HER2$^+$ and HER2/CEP17 ratio <2.0 who underwent surgery after 2007. The European Institute of Oncology ethical committee approved the present analysis. In the study we included tumor samples from patients who gave their consent to use their biological samples for research purpose. HER2 status was assessed according to 2013 recommendations of American Society of Clinical Oncology and College of American Pathologists guidelines. We used the HercepTest preparation kit (Dako, Glostrup, Denmark) and scored the results according to FDA/EMA recommendations; therefore, score 2+ cases were those exhibiting mild to moderate complete membranous staining in at least 10% of cells. IHC data on estrogen receptor (ER) and progesterone receptor (PR) expression and on proliferation index (Ki-67) were

Table I. *Patients' characteristics*.

Variable	Levels	N (%)	HER2/CEP 17 ratio Geometric Mean (95% CI)	*p*-Value
All patients		455 (100)	1.25 (1.22-1.27)	
Age class	<35	22 (4.8)	1.20 (1.12-1.29)	0.19
	35-50	197 (43.3)	1.22 (1.19-1.26)	
	51-65	178 (39.1)	1.27 (1.23-1.31)	
	>65	58 (12.7)	1.29 (1.22-1.36)	
Menopausal status	Pre-menopausal	220 (48.4)	1.21 (1.18-1.24)	0.0032
	Post-menopausal	235 (51.6)	1.28 (1.25-1.32)	
Histology	Ductal	384 (84.4)	1.25 (1.23-1.28)	0.27
	Others	71 (15.6)	1.22 (1.16-1.28)	
pT	1	244 (53.6)	1.25 (1.22-1.29)	0.049
	2	172 (37.8)	1.26 (1.22-1.30)	
	3-4	39 (8.6)	1.15 (1.09-1.22)	
No. of positive lymph nodes	None	236 (51.9)	1.25 (1.21-1.28)	0.32
	1-3	141 (31)	1.26 (1.22-1.31)	
	4+	78 (17.1)	1.21 (1.16-1.26)	
Grade	Unknown*	8 (1.8)	1.14 (1.02-1.28)	
	1-2	279 (61.3)	1.23 (1.20-1.26)	0.051
	3	168 (36.9)	1.28 (1.24-1.32)	
ER/PgR	PgR=0 and ER = 0	35 (7.7)	1.26 (1.16-1.36)	0.64
	0<PgR<50 or 0<ER<50	161 (35.4)	1.26 (1.22-1.30)	
	ER>=50 and PgR >=50)	259 (56.9)	1.24 (1.21-1.27)	
Ki-67	<14%	69 (15.2)	1.15 (1.11-1.21)	0.0008
	>=14%	386 (84.8)	1.26 (1.24-1.29)	
PVI	Absent	272 (59.8)	1.23 (1.20-1.26)	0.15
	Moderate/Focal	94 (20.7)	1.28 (1.23-1.34)	
	Diffuse	89 (19.6)	1.26 (1.21-1.32)	

obtained from the original pathology reports. FISH was performed according to the manufacturer's instructions with probes for HER2 and CEP17 (Abbott Molecular Diagnostics, Abbott Park, IL, USA), as described previously. For analysis, 10 invasive areas on each slide were selected and automatically acquired at ×40 with the motorized Metafer scanning system (Carl Zeiss MetaSystems GmbH, Jena, Germany) and Axio Imager epifluorescence microscope (one focus plane for DAPI [4',6-diamidino-2-phenylindole] and 13 focus planes for green and red spots). PathVysion V2 software (MetaSystems Hard & Software GmbH, Altlußheim, Germany) (FDA approved) was used to automatically analyze HER2 and CEP17 probes. A range of 300-800 cells was examined. Results were reviewed by three pathologists and scored according to both FDA/EMA recommendations (7, 8) and ASCO/CAP guidelines 2013 for dual-signal assay analyses. In addition, we also simulated the scoring for the single-signal assay by taking into account HER2 gene counting only. In the latest ASCO/CAP 2013 guidelines, *HER2* gene counting is the only parameter evaluated for single-probe ISH and is also integrated into the evaluation of the results of the HER2/CEP17 ratio for dual-signal ISH. The distribution of FISH ratio is reported in Figure 1. Heterogeneity was defined according to the supplemental material shown in the ASCO/CAP 2013 guidelines. Since the HER2/CEP17 ratio was positively skewed, it was analyzed after logarithmic transformation. The association between HER2/CEP17 ratio and other known prognostic factors was evaluated by using linear regression. The role of HER2/CEP17 ratio on recurrence-free survival was assessed with univariable and multivariable Cox regression models.

Results

Patients characteristics are reported in Table I. Fifty-one percent were node negative, 51% were postmenopausal, 92% had ER-positive BC and 85% had Ki-67 ≥14%. The overall geometric mean of HER2/CEP17 ratio was 1.25 (95%CI=1.22-1.27). A highly significant positive relationship between HER2/CEP17 ratio and Ki-67 was observed ($p<0.001$). Four-hundred patients (80%) received an endocrine treatment (99 patients in combination with chemotherapy) as adjuvant treatment. Thirty-six patients received only chemotherapy. At a median follow-up of 2.7 years, 40 tumor recurrences were observed [15 locoregional events and 25 distant metastases]. Overall, in patients with IHC HER2 2+ early BC HER2/CEP17 ratio was not associated with the risk of recurrence, both at univariate (Figure 2) and multivariate analysis. In an explorative subgroup analysis, a significant interaction between HER2/CEP17 ratio and nodal involvement was observed ($p=0.02$). Among patients with node-negative

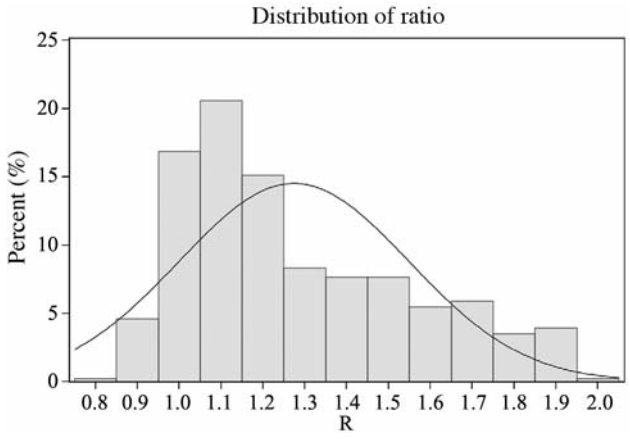

Figure 1. *Distribution of FISH Ratio.*

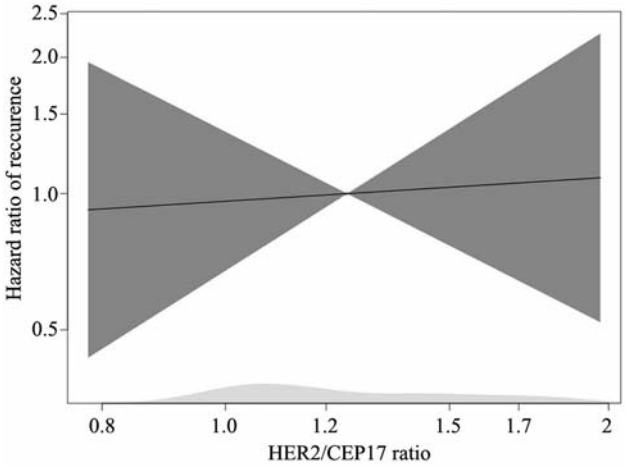

Figure 2. *Effect of HER2/CEP17 ratio, treated as a continuous variable in a univariable Cox model, on the hazard ratio (HR) of tumor recurrence. The reference HER2/CEP17 ratio used for the computation of HR is the overall geometric mean (1.25).*

disease, those with high HER2/CEP17 ratio (>1.25, *i.e.* the overall observed geometric mean] were at higher risk of recurrence as compared to patients with low HER2/CEP17 ratio (adjusted HR=4.8, 95%CI=1.3-17.8).

Discussion

This study reports data from a large, mono-institutional cohort of patients with HER2 equivocal early breast cancer. Whether patients with IHC HER2 equivocal tumors should receive targeted therapy remains a challenging question for oncologists, particularly as reporting guidelines evolve. A clinical note in 2011 regarding ASCO/CAP guideline recommendations on HER2 testing stated that the expert panel did not recommend withholding treatment for patients with an equivocal HER2 test whose results fell within ranges for eligibility in the first generation adjuvant HER2-targeted trials. The 2013 ASCO/CAP guidelines maintain this caveat, giving latitude to oncologists to consider HER2-targeted therapy for patients with equivocal HER2 test results, even after reflex testing with an alternative assay. Clearly, there is a need to further examine this group of tumors to obtain optimal prognostic and treatment determinations. A randomized phase III trial of adjuvant therapy comparing chemotherapy alone (6 cycles of docetaxel plus cyclophosphamide or 4 cycles of doxorubicin plus cyclophosphamide followed by weekly paclitaxel) to chemotherapy plus trastuzumab in women with node-positive or high-risk node-negative HER2-Low invasive breast cancer is actually ongoing (study B47 of the National Surgical Adjuvant Breast and Bowel Project [NSABP] study group). Eligibility includes node positive or high-risk node-negative BC patients with HER2 IHC 1+ or 2+ scores, but non-amplified by FISH; 3,260 patients will be enrolled. A very limited number of studies assessed the role of HER2 equivocal status in early breast cancer (9). The largest series analyzed was published by Sapino *et al*. (9). A cohort of 957 immunohistochemistry-evaluated HER2-equivocal cases was analyzed by dual-color FISH. The results were assessed according to U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines and American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) 2007 and 2013 guidelines for dual- and single-signal *in situ* hybridization (ISH) assays. HER2 amplification varied from 15% (ASCO/CAP 2007 HER2/CEP17 ratio) to 29.5% (FDA/EMA HER2 copy number). According to the ASCO/CAP 2013 interpretation of the dual-signal HER2 assay, ISH-positive carcinomas accounted for 19.7%.

In contrast with the ASCO/CAP 2007 ratio, this approach labeled as positive all 32 cases (3.34%) with a HER2/CEP17 ratio <2 and an average HER2 copy number ≥6.0 signals per cell. In contrast, only one case showing a HER2 copy number <4 but a ratio ≥2 was diagnosed as positive. Authors concluded that the ASCO/CAP 2013 guidelines seem to improve the identification of HER2-positive carcinomas (9). In our study, despite the samples size in not similar, we provide data on outcome of patients with HER2 equivocal status. Our results do not support indication to trastuzumab in patients with equivocal IHC HER2 early breast cancer. In summary, in our series HER2 equivocal BC represent a subgroup of high-grade estrogen receptor-positive tumors with higher proliferative activity. The B47 NSABP study will assess whether the addition of trastuzumab to chemotherapy improves invasive disease-free survival [IDFS] in this group of patients.

Conclusion

We found no significant association between risk of recurrence and HER2 equivocal testing in patients with early breast cancer. In subgroup analysis, a significant interaction between HER2/CEP17 ratio and nodal involvement was observed (p=0.02). Patients with HER2 equivocal status have no significant risk of recurrence. In our analysis IHC HER2 2+ status with HER2/CEP17 ratio <2.0 has no impact on prognosis of patients with early breast cancer.

Ethics Approval and Consent to Participate

Approval was obtained from the European Institute of Oncology Ethics Committee to perform this study. All patients included in the analysis consented to use their tumor samples and their data for research purpose

Conflicts of Interest

The Authors have no conflict of interest to declare.

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