HER2 staining intensity in HER2-positive disease: relationship with FISH amplification and clinical outcome in the HERA trial of adjuvant trastuzumab

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Background: Trastuzumab treatment improves survival of HER2-positive primary breast cancer. HER2 staining intensity varies widely in HER2-positive tumours.

Patients and methods: We investigated whether differences in immunohistochemical (IHC) staining intensity for HER2 in HER2-positive tumors (IHC 3+ or FISH ratio ≥2.0) was associated with prognosis or benefit from trastuzumab treatment in patients randomized to 1 year or no trastuzumab in the HERceptin Adjuvant (HERA) trial. Median follow-up was 2 years. The nested case–control analysis, included 425 patients (cases) with a disease-free survival (DFS) event and two matched controls (no DFS event) per case. Tissue sections stained for HER2 were assessed for HER2 staining intensity by image analysis.

Results: HER2 staining intensity varied widely and correlated with HER2 gene copy number (Spearman, r = 0.498, P < 0.001) or less closely with HER2/CEP17 FISH ratio (r = 0.396, P < 0.001). We found no significant difference in DFS in the observation arm according to staining intensity (odds ratio [OR] change per 10 unit change in intensity: 1.015, 95% confidence interval [CI] 0.930–1.108) and no impact of staining intensity on benefit derived from 1-year trastuzumab (OR: 1.017, 95% CI 0.925–1.120).

Conclusions: Variability in HER2 staining in HER2-positive tumours has no role in clinical management with adjuvant trastuzumab.

HERA trial no: NCT00045032.

Key words: breast cancer, HER2, IHC intensity, HERA, Trastuzumab

introduction

Approximately 15% of newly diagnosed invasive breast carcinomas that overexpress HER2 and/or show HER2 gene amplification are associated with poorer prognosis [1]. HER2 status is an essential factor in selecting patients for treatment with trastuzumab, a recombinant humanized monoclonal antibody against the HER2 protein.

The diagnosis of HER2 overexpression is most commonly based on two approaches used in conjunction: immunohistochemistry (IHC) and in situ hybridization (ISH). IHC reveals overexpression of HER2 protein on the cell membrane. The degree of staining is most frequently described on a scale of 0–3, with 3+ being considered unequivocally positive (>10% of tumour cells with intense circumferential membrane staining as per the FDA approved cutoff, or >30% immunostained tumour cells according to more recent American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendations) [2], 2+ equivocal and 0/1+ negative. ISH reveals the number of HER2 gene copies per cell and has been most commonly conducted with a fluorescent (FISH) probe. A second probe labeling the centromeric region of chromosome 17 (CEP17) is often used allowing the ratio of HER2/CEP17 ratio to be calculated. In clinical trials, the ratio of 2.0 or greater has been regarded as FISH-positive. However, an unambiguous cutoff of 2.2 was proposed more recently in the ASCO/CAP guidelines [2].

The degree of HER2 staining intensity varies markedly in tumours defined as positive by the above criteria (Figure 1). In the HERA and N9831 trials, there were no marked differences in benefit from trastuzumab treatment according to the degree of amplification [3, 4]. We have now investigated whether HER2
IHC staining intensity was associated with prognosis or benefit from treatment in the 1-year trastuzumab and observation arms of the HERA trial [5]. We also assessed the degree to which staining intensity correlated with HER2 gene copy number versus HER2:CEP17 ratio.

**methods**

**patients**

The HERA (Breast International Group [BIG] 01-01) trial is an international, multicenter phase III randomized trial involving women with HER2-positive (overexpressed or amplified) early-stage invasive breast cancer and has been described in detail elsewhere [5]. The trial compares 1 year and 2 years of trastuzumab versus no trastuzumab in patients who completed locoregional therapy (surgery plus radiotherapy where indicated) and a minimum of four courses of chemotherapy.

In this nested case–control study, we defined cases as patients with a disease-free survival (DFS) event (defined in [5]). We considered patients randomized to 1-year trastuzumab and observation and the database of 14 February 2006 after a median follow-up of 2 years, when there was little impact of selective crossover from the observation to the 1-year trastuzumab arm [6].

**HER2 status assessment**

Staining intensity was assessed on slides used to confirm the patients’ tumour HER2 status in the HERA trial central laboratory (Targos, Kassel, Germany). A result on IHC (DAKO, HercepTest) of 3+ (>10% stained cells) was required for tumours assessed by the participating institution as 3+, and a positive result from FISH (PathVysion, Vysis; HER2:CEP17 ratio ≥2.0) was required for tumours that were assessed in the participating institution as IHC2+ or FISH-positive. Additional FISH values were available as previously described [3].

Immunohistochemical HER2 staining of tissue sections was carried out manually using the HercepTest (DAKO) according to the manufacturer’s instructions with the exception that Epitope retrieval occurred at 95–99°C using an oil bath in order to guarantee stable temperature conditions. In addition to the kit cell culture controls, internal breast cancer tissue controls were used.

**image acquisition**

Images of tissue sections were collected in the central laboratory using the Ariol image analysis system (Leica Microsystems (Gateshead) Ltd, UK) equipped with an Olympus BX61 microscope, and a black and white camera (MegaPlus ES 4.0/E, Redlake). Slides were scanned using the Hersight2 assay initially at ×1.25 objective and then to capture images using ×20 objective.

**image analysis**

Analysis of HER2 staining intensity took place at the Royal Marsden Hospital using an identical Ariol image analysis system to the central laboratory.

Representative invasive breast cancer areas in each section image were selected. If a limited number of invasive tumour cells was present, the entire invasive component was selected. If invasive tumour was present in abundance, 8 or 9 regions (single capture frame size ~750 µm²) representing HER2 staining intensity variability of the examined section were selected. In each region, an equal number of invasive tumour areas was marked manually to avoid including any normal tissue in membrane staining intensity measurements.

The staining intensity of up to 100 areas per tumour was recorded. The mean membrane intensity of all representative areas selected for analysis was used as a measurement of HER2 staining intensity. The minimum and maximum intensity from the representative areas was also recorded.

**statistical analysis**

In nested case–control studies [7], cases of an event of interest in a defined cohort of patients are identified, and for each, a specified number of matched controls is selected from those who do not have the event of interest by the time the event occurred in the case. The occurrence of the event of interest is random with probability dependent on covariates. In this analysis, the cohort of patients was randomized to 1-year Herceptin and observation, and the event of interest was the occurrence of a DFS event. There were 425 case–control ‘triplets’ of a case matched with two controls. The same patient could be a control for more than one case and a control patient could later become a case. Among the 850 controls selected, there were 726 unique controls and 646 unique controls that did not later become a case.

**correlation between FISH and HER2 intensity**

For the case–control triplets, the difference between the log of the HER2 copy number, the log of the FISH ratio, and staining intensity among the case and the controls was calculated. The difference in the log of the HER2 copy number versus the difference in staining intensity and the difference in log of the FISH ratio versus the difference in staining intensity were plotted. The log of the HER2 copy number and log of the FISH ratio were considered in order to give a near linear relationship. The Spearman correlation coefficients were calculated.

**relationship of HER2 staining intensity with clinical outcome**

Conditional logistic regression [8] (also see Online Appendix) of the probability of being a case was carried out among the case–control triplets with the following covariates: staining intensity, randomized arm,
menopausal status at randomization, nodal status, estrogen receptor (ER) status and the interaction term for staining intensity and randomized arm (referred to as model 4 in supplementary Table S1, available at *Annals of Oncology* online). The stratification factor of age group was not considered in the conditional logistic regression model due to relationship with menopausal status. The stratification factor of (neo)adjuvant chemotherapy did not add any information to the conditional logistic regression. The odds ratio change per 10-unit increase in staining intensity and 95% confidence interval (CI) was calculated by randomized arm. Statistical analysis was carried out in SAS version 9.2.

**results**

HER2 intensity measurements (Figure 1) were available on 381 of 425 (89.6%) cases and 755 of 850 (88.8%) controls (exclusions: poor image quality, errors during image data transfer, insufficient invasive tumour content for image analysis; nine cases were excluded because of extreme variability in staining intensity that could have been explained by either variability in staining efficiency or clonal HER2 expression). A consort diagram showing the patient flow through the study is shown in supplementary Figure S1, available at *Annals of Oncology* online.

The mean HER2 staining intensity of individual tumours ranged from 75 to 209 units with a peak between 170 and 180 (Figure 2A). Figure 2B illustrates the within tumour variability in HER2 staining intensity of 30 randomly selected tumours. The intensity of each invasive tumour areas is shown as a black spot and the mean intensity as a red square. The standard deviation (SD) of the range of values varied between tumours from 4.0 to 17.5 and the median SD was 9.1.

**relationship between staining intensity and FISH ratio and HER2 copy number**

There were highly significant correlations between HER2 staining intensity and HER2 copy number across all tumours in the case–control study (Spearman Correlation, $r = 0.498$, $P < 0.001$) (Figure 3A) and in separate case and control populations (Spearman’s correlation, $r = 0.414$, $P < 0.001$ and $r = 0.535$, $P < 0.001$, respectively). These relationships with staining intensity were weakened when the correlation was assessed with HER2/CEP17 FISH ratio ($r = 0.396$, 0.306 and 0.440, respectively) (Figure 3B). As expected, the image analysis intensity for tumours scored visually as IHC 2+ was overall lower than that for IHC 3+ tumours, but there was overlap in intensity among the cohorts (Figure 3A and B).

**relationship between HER2 staining intensity and benefit from trastuzumab**

As noted, staining intensity was not available for all patients in the case–control triplets. One case–control triplet was excluded because the ‘case’ patient has a protocol violation of unknown ER status. Therefore, conditional logistic regression was carried out on 297 case–control triplets using the covariates referred to as model 4 in supplementary Table S1 and S2, available at *Annals of Oncology* online. Figure 4B and supplementary Table S3, available at *Annals of Oncology* online show the odds ratios change per 10-unit change in staining intensity. We found no difference in DFS in the observation arm according to staining intensity (odds ratio change per 10 unit change in intensity: 1.015, 95% CI 0.930–1.108). HER2 staining intensity also had no apparent impact on benefit derived from 1-year trastuzumab (odds ratio change per 10 unit change in intensity: 1.017, 95% CI 0.925–1.120; ‘P-value for interaction for staining intensity’.
intensity and randomized arm \( = 0.9735 \). Similarly, neither maximum nor minimum staining intensity was related to DFS in the observation arm or benefit from trastuzumab (Figure 4A and C, respectively).

**discussion**

Trastuzumab treatment of both early-stage and advanced/metastatic HER2-positive breast cancer patients leads to major
improvements in outcome, including improved survival [5, 9, 10]. The HERA trial has recently reported that patient benefit from 2 years’ trastuzumab is similarly to that from 1 year; thus 1 year of trastuzumab as investigated in the current study remains the standard of care. Other than a small recent set of data, the indications are that trastuzumab is only effective in disease that overexpresses the HER2 protein and/or shows gene amplification [2]. For ER, the other major biomarker in breast cancer, there is a greater benefit of tamoxifen for tumours with the highest expression of the target among those considered ER-positive [11]. We, and others, have previously reported that there is no evidence for such a quantitative relationship between trastuzumab benefit and levels of HER2 when the latter was assessed according to the degree of gene amplification among HER2+ patients [3, 4]. In the current study, we asked whether an assessment of HER2 staining intensity as an approximation of target expression at a protein level was related to outcome given that antibody binding to cell surface HER2 is the key event in trastuzumab pharmacology.

Strengths of the study include the assessment of slides from a large registration standard trial and the application of a widely used FDA-approved staining method in a central laboratory with high-quality standards. Limitations include the assessment of protein expression by immunohistochemistry, which does not show a linear read-out in relation to expression levels. The use of a technique such as AQUA, which is based on fluorescence, may have improved linearity [12] but could have been undertaken on only the about 20% of patients in the HERA trial from whom blocks for research were available. The assessment of conventional immunohistochemistry also meant that a positive finding could have been of immediate relevance to the large majority of laboratories using similar methodology.

The relationship between gene amplification and staining intensity indicates that staining level has substantial biological meaning and is not just the result of analytical variability. The absence of a relationship of HER2 staining intensity with outcome in terms of DFS in the observation (no trastuzumab) arm or in degree of benefit from trastuzumab indicates that any relationship between protein expression and these clinical end points cannot be strong in HER2-positive tumours. It should not be assumed that the same would hold for other HER2-targeted therapies where pharmacodynamics may vary from those with trastuzumab.

While the relationship with clinical outcome was the main goal of this project, a number of other issues of importance to current clinical research were addressed. The extensive assessment of staining intensity in up to 100 areas per tumour provided information on the degree of heterogeneity (Figure 2). While it is clear that there was some variability, other than in the nine tumours excluded because of extreme variability in staining intensity possibly due to clonal expression, for most tumours, the range was relatively narrow with no regions in over 90% of the tumours showing regions that could have been considered negative. Heterogeneity of staining intensity may appear greater when evaluated visually and therefore subjectively against the background of other highly stained areas rather than by the objective method of image analysis. There was no significant relationship between clinical outcome and either minimum or maximum staining intensity. Our data therefore do not lend support to the concept that HER2-negative clones commonly exist in HER2-positive tumours and provide a frequent means of escape from treatment with antiHER2 therapies.

The indication of a closer relationship between HER2 staining intensity and gene copy number as opposed to the HER2/CEP17 ratio suggests that the creation of this ratio worsens rather than improves the biological relevance of amplification. This concurs with the recent report that true polysomy of chromosome 17 is uncommon and that the creation of the ratio with a probe to CEP17, which itself is subject to amplification, may falsely ‘correct’ for polysomy [13, 14]. Gene copy number may, therefore, be the preferred means of describing HER2 amplification.

There is considerable interest in the cross-talk between ER and HER2 [15, 16]. It is well known that positivity rates for HER2 are lower in ER-positive tumours than in ER-negative and among those HER2-positive tumours that are ER-positive HER2 expression levels are lower than in ER-negative tumours [17]. It is of note, however, that this analysis indicated that the relationship between HER2 gene copy number and staining intensity was similar for both ER-positive versus ER-negative tumours.

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disclosure

MD and JR have acted on Roche International Herceptin Advisory Board. MD is currently conducting research for which Roche has provided a grant. MP received consultancy honoraria from Roche-Genentech. MP’s institution received funding from Roche in respect to the HERA trial. Other authors have declared no conflicts of interest.

references

Circulating tumor cells predict progression-free and overall survival in Chinese patients with metastatic breast cancer, HER2-positive or triple-negative (CBCSG004): a multicenter, double-blind, prospective trial†


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Background: The aim of this multicenter, double-blind, prospective study was to evaluate the potential utility of circulating tumor cell (CTC) measurements in predicting responses to anticancer therapies, including response to human

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