Very high quantitative tumor HER2 content and outcome in early breast cancer


1Department of Oncology, Helsinki University Central Hospital and University of Helsinki, Helsinki, Finland; 2Division of Research and Development, Monogram Biosciences, Inc., South San Francisco, USA; 3Pharma, Turku, Finland; 4Division of Clinical Research, Monogram Biosciences, Inc., South San Francisco, USA; 5Department of Oncology, Kuopio University Hospital, Kuopio, and Vaasa Central Hospital, Vaasa; 6Department of Oncology, Kanta-Häme Central Hospital, Hämeenlinna; 7Department of Oncology and Radiotherapy, Oulu University Hospital, Oulu; 8Department of Oncology, Turku University Central Hospital, Turku; 9Laboratory of Cancer Biology, Institute of Medical Technology, University of Tampere and Tampere University Hospital; 10Department of Oncology, Tampere University Hospital, Tampere, Finland

Received 27 June 2010; revised 8 November 2010; accepted 12 November 2010

**Background:** It is unknown how a very high tumor total HER2 (human epidermal growth factor receptor-2) content (H2T) influences outcome in early breast cancer treated with adjuvant trastuzumab plus chemotherapy.

**Patients and methods:** H2T was measured using a novel quantitative assay (HERmark®) from formalin-fixed tumor tissue of 899 women who participated in the FinHer trial (ISRCTN76560285). In a chromogenic in situ hybridization (CISH) test, 197 (21.9%) patients had HER2-positive cancer and were randomly assigned to receive trastuzumab or control.

**Results:** Cancer H2T levels varied 1808-fold. High H2T levels were correlated with a positive HER2 status by CISH (P < 0.0001). A nonlinear association was present between H2T and the hazard of distant recurrence in a subpopulation treatment effect pattern plot analysis in CISH-positive disease. Patients with very high H2T (defined by ≥22-fold the median of HER2-negative cancers; 13% of CISH-positive cancers) did not benefit from adjuvant trastuzumab [hazard ratio (HR) 1.23; 95% confidence interval (CI) 0.33–4.62; P = 0.75], whereas the rest of the patients with HER2-positive disease by CISH (87%) did benefit [HR 0.52; 95% CI 0.28–1.00; P = 0.050].

**Conclusion:** Patients with HER2-positive breast cancer with very high tumor HER2 content may benefit less from adjuvant trastuzumab compared with those whose cancer has more moderate HER2 content.

**Key words:** adjuvant therapy, breast cancer, human epidermal growth factor receptor-2, in situ hybridization, prognosis, trastuzumab

**Introduction**

Human epidermal growth factor receptor-2 (HER2) confers adverse prognosis in breast cancer [1] and is a predictor of clinical responsiveness to treatment with the humanized mAb trastuzumab and other HER2-targeting agents [2]. Tumor HER2 positivity is usually assessed using immunohistochemistry (IHC), which measures HER2 protein expression, or using FISH or chromogenic in situ hybridization (CISH), which measure HER2 gene copy numbers. Patients whose cancer is scored HER2 positive by these standard techniques have better outcomes on trastuzumab than those whose cancer is scored negative, but the differences in the degree of positivity by these methods have not been shown to discriminate between groups of patients with different outcomes [3]. In general, cancers scored 2+ for HER2 expression by IHC require a confirmatory FISH or CISH test in order to determine patient eligibility for trastuzumab treatment [4, 5].

The HERmark® (Monogram Biosciences Inc., South San Francisco, CA) proximity assay uses a multiple antibody approach to make precise quantitative measurements of formalin-fixed paraffin-embedded tumor HER2 content with greater sensitivity than IHC and over approximately a 1000-fold dynamic range [6]. When breast cancer HER2 protein expression was measured using the HERmark® assay, patients with advanced disease whose cancer was HER2 positive by FISH but low total HER2 protein content (H2T) by HERmark® had a similar outcome following treatment with trastuzumab as those whose cancer was FISH negative and H2T low [7]. Using this novel assay, we found in a series of patients with advanced breast cancer that patients whose cancer was both HER2 FISH positive and expressed the highest levels of...
H2T had similar outcome to those whose cancer was FISH negative and expressed low levels of H2T [8].

In the present study, we examine the effect of the primary tumor H2T levels on outcome. To our knowledge, a similar analysis has not been previously carried out in the setting of early breast cancer.

**Patients and Methods**

**Study Population and Tumor Tissue Samples**

In the FinHer trial (identifier ISRCTN76560285), 1010 women diagnosed with either node-positive breast cancer or node-negative cancer with a primary tumor >20 mm and negative in immunostaining for the progesterone steroid hormone receptor were randomly assigned to receive either three cycles of weekly vinorelbine (25 mg/m²) followed by three 3-weekly cycles of docetaxel (100 mg/m²), epirubicin (60 mg/m²), and cyclophosphamide (600 mg/m²; FEC) or to three 3-weekly cycles of fluorouracil (600 mg/m²), epirubicin (60 mg/m²), and cyclophosphamide (600 mg/m²; FEC) or to three 3-weekly cycles of docetaxel (100 mg/m²) followed by 3-weekly cycles of fluorouracil (600 mg/m²), epirubicin (60 mg/m²), and cyclophosphamide (600 mg/m²; FEC) [9]. No patient was lost to follow-up.

A benefit for those patients assigned to docetaxel and to those assigned to trastuzumab [10–12] had improved outcome compared with controls [9], a finding also observed in the FinHer trial carried out after a median of 62 months of follow-up at the Clinical Laboratory Improvement Amendments, and all tissue analyses were carried out in a College of American Pathologists-certified clinical reference laboratory at Monogram Biosciences, Inc. (South San Francisco, CA). The results were transmitted to the FinHer investigators (Helsinki University Central Hospital, Helsinki, Finland) for statistical analysis. Investigators at Monogram Biosciences were blinded to clinical outcome data at all times.

**IHC and Central CISH Testing**

Detection of HER2 protein was first carried out by IHC according to the guidelines of each participating institution. When HER2 expression was scored either 2+ or 3+ on a scale of 0, 1+, 2+, or 3+, CISH was then carried out in one of the two reference laboratories [9, 13]. Tumors with six or more copies of HER2 or with gene clusters in CISH were classified HER2 positive. A chromosome 17 pericentromeric probe was used in equivocal cases. All IHC and CISH testing was done before HERmarkTM or HER2 amplification did not participate for randomization for the present analysis. Of these, 702 (78.1%) were classified as HER2 positive by IHC and CISH before patient entry to the FinHer trial (Figure 1). One patient who had tumor with HER2 amplification did not participate for randomization for trastuzumab. The study patients provided a written permission to use clinical data and tumor tissue for research purposes, and the study was approved by an ethics committee (HUS/6/106/2007).

The current study is based on the final analysis of the patients and methods. When HER2 expression was scored either 2+ or 3+ on a scale of 0, 1+, 2+, or 3+, CISH was then carried out in one of the two reference laboratories [9, 13]. Tumors with six or more copies of HER2 or with gene clusters in CISH were classified HER2 positive. A chromosome 17 pericentromeric probe was used in equivocal cases. All IHC and CISH testing was done before HERmark™ assay performance. Whenever possible, the proximity assay was done using sections from the same tissue block that had been used for a CISH assay.

We also carried out central IHC for HER2 expression for the present 899 samples. This was done at PhenoPath Laboratories (Seattle, WA) using the same tissue blocks as for H2T testing. In central IHC retesting, the samples were scored using the Dako polyclonal A0485 antibody and validated procedures [16]. The samples were classified using the scale of 0, 1+, 2+, or 3+, as well as the histoscore, obtained by multiplying the percentage of HER2-positive cancer cells in the sample by the staining intensity ranging from 0 to 3+ [17]. Thus, the maximum histoscore was 300 (all tumor cells stained strongly for the HER2 protein) and the minimum score was 0 (none of the tumor cells expressed HER2).

**Statistical Methods**

Statistical analyses were carried out using the SAS System for Windows (SAS Institute Inc., Cary, NC) package by a professional statistician (ML) at 4Pharma (Turku, Finland) according to a pre-specified statistical analysis plan. The primary end point was distant recurrence-free survival (RFS), which was calculated from the date of randomization to the date of first diagnosis of distant breast cancer recurrence (with histologic or cytoplasmic confirmation or with radiologic evidence) or to death, whichever occurred first. RFS was computed from the date of randomization to first breast cancer recurrence (local, distal, or contralateral invasive breast cancer) or to death, whichever occurred first. Overall survival (OS) was computed from the date of randomization to the date of death.
Survival between groups was compared using the Kaplan–Meier life table method and unstratified Cox proportional hazards model with the treatment group or the risk group as the only covariate in the model, unless stated otherwise. Subpopulation treatment effect pattern plot (STEPPE) analyses [18] using the sliding window approach were applied to illustrate the correlation between the survival outcome (either a hazard ratio [HR] between two groups or a hazard rate within a group) and H2T as a continuous variable. To identify optimal cut-off values for H2T, a positional scanning analysis was subsequently carried out by scanning through all possible cut-off values (i.e. from the minimum to the maximum H2T) and calculating the HRs for the subset of patients above each possible cut-off value. The HRs were plotted as a function of the cut-off values.

Distributions of categorical variables were compared with chi-square test, continuous variables with Mann–Whitney test and correlations by computing the Spearman correlation coefficient. All P values are two sided and not adjusted for multiple testing.

**Results**

The patient and tumor characteristics of the 899 cases analyzed are provided in supplemental Table S1 (available at Annals of Oncology online). Tumor H2T levels varied greatly, by 1808-fold (Table 1). High tumor H2T content was strongly correlated with a positive tumor HER2 status defined originally using CISH (P < 0.0001), but there was, however, substantial overlap between the H2T distributions of the HER2-positive and -negative groups defined by original IHC followed by CISH (Figure 2A). All those 131 tumors that were CISH positive and -negative groups defined by original IHC followed by CISH (Figure 2A). All those 131 tumors that were CISH positive and also 3+ on a central IHC retest had H2T content higher than the median H2T value of the cases that were HER2 negative by CISH, but H2T still distributed over a 1.9 log (69-fold) range in this subset (Figure 2B). When semiquantitative IHC histoscores for this HER2-positive subset were generated, all tumors except one outlier with a histoscore of 220 fell within the histoscore range of 250–300, while the range in the H2T values was much greater (1.9 logs).

When tumor H2T content was examined for correlation with three survival end points, RFS, distant RFS, and OS among women with HER2-negative cancer in Cox models that included randomization for trastuzumab versus control had a tumor H2T value of the cases that were HER2 negative by CISH, but H2T still distributed over a 1.9 log (69-fold) range in this subset (Figure 2B). When semiquantitative IHC histoscores for this HER2-positive subset were generated, all tumors except one outlier with a histoscore of 220 fell within the histoscore range of 250–300, while the range in the H2T values was much greater (1.9 logs).

When tumor H2T content was examined for correlation with three survival end points, RFS, distant RFS, and OS among women with HER2-negative cancer in Cox models that included trastuzumab treatment as a categorical variable and tumor H2T content or log H2T as a continuous variable as covariates, no statistically significant relationships were observed with outcome, and no interaction between treatment and H2T or log H2T was detected (P = 0.81 and 0.74). Since the association between H2T and outcome may not be linear, and nonproportional associations may not be detected in a Cox proportional hazards model, we carried out a STEPP analysis and examined the variation in the HR between the trastuzumab and control groups as a function of tumor H2T content (Figure 3). The further the curve is below an HR of 1.0 in this analysis, the greater the reduction of risk for those patients in the trastuzumab group compared with patients in the control arm who have similar tumor H2T content. The STEPP analysis suggests that the HR approaches 1.0 at the upper end of the H2T distribution, implying the possible existence of a subpopulation of patients with very high levels of HER2 expression who may derive limited benefit from treatment with trastuzumab (Figure 3A). When the hazard rates for the trastuzumab and control arms were examined separately as a function of H2T using the STEPP analysis, a rise in the hazard rate was observed at the upper end of the H2T distribution particularly in the group of patients who received adjuvant trastuzumab (Figure 3B). Although a STEPP analysis can be very useful in detecting nonlinearity in variable–outcome relationships, it is not an appropriate tool for defining the boundaries of subpopulations that may exist within the overall cohort. Therefore, we carried out positional scanning analyses to investigate whether there was a particular cut-off above which one could observe a change in the relationship between H2T and survival. The results were consistent regardless of whether distant RFS (Figure 4A) or OS (Figure 4B) was selected as the end point. In these analyses, the HR represents the relative risk of trastuzumab-treated patients versus the controls for all those subjects who have tumor H2T content above the cut-off. Thus, the subset of patients with tumor H2T values higher than approximately log H2T = 2.1 had an HR that reached and exceeded 1.0 suggesting that patients whose tumor contained very high amounts of HER2 protein derived little or no benefit from adjuvant trastuzumab. Twenty-five (13%) of the 196 patients with HER2-positive cancer and who participated in randomization for trastuzumab versus control had a tumor H2T value higher than log H2T = 2.1. This cut-off corresponds to ~22-fold higher tumor HER2 protein content compared with the median HER2 content found in tumors defined as HER2 negative by CISH (Figure 2A) and to approximately twofold higher tumor HER2 content compared with the

### Table 1. Tumor quantitative HER2 protein content and histoscore values of 899 breast cancers

<table>
<thead>
<tr>
<th>HER2 measure</th>
<th>Total population</th>
<th>HER2-negative cancer by CISH (n = 702)</th>
<th>HER2-positive cancer by IHC and CISH (n = 197)</th>
<th>HER2-positive cancer by IHC and CISH (n = 131)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2T, median (range)</td>
<td>7.0 (0.4–721.2)</td>
<td>5.7 (0.4–118.4)</td>
<td>49.7 (3.8–721.2)</td>
<td>68.8 (10.4–721.2)</td>
</tr>
<tr>
<td>H2T, 25th and 75th percentiles</td>
<td>4.3, 15.7</td>
<td>3.8, 9.4</td>
<td>23.4, 89.6</td>
<td>43.8, 109.8</td>
</tr>
<tr>
<td>Log10 H2T, median (range)</td>
<td>0.8 (−0.4 to 2.9)</td>
<td>0.8 (−0.4 to 2.1)</td>
<td>1.7 (0.6–2.9)</td>
<td>1.8 (1.0–2.9)</td>
</tr>
<tr>
<td>Histoscore*, median (range)</td>
<td>60 (0–300)</td>
<td>20 (0–300)</td>
<td>296 (0–300)</td>
<td>295 (220–300)</td>
</tr>
</tbody>
</table>

*Histoscore was obtained by multiplying the percentage of HER2-positive cancer cells in the sample by the staining intensity ranging from 0 to 3+: [histoscore = (% at 0) × 0 + (% at 1+) × 1 + (% at 2+) × 2 + (% at 3+) × 3].

HER2, human epidermal growth factor receptor-2; CISH, chromogenic in situ hybridization; IHC, immunohistochemistry; H2T, tumor total HER2 content.
median of those tumors that were deemed HER2 positive both by centrally carried out IHC and CISH (Figure 2B). The effect of categorized log H2T ($\leq 2.1$ versus $>2.1$) on the hazard of RFS was significant ($P = 0.046$) for the trastuzumab group in the analysis shown in Figure 3B and nonsignificant ($P = 0.77$) for the control group.

We next examined the effect of tumor H2T content on distant RFS using the positional scanning analyses-derived cut-off and Kaplan–Meier life table analyses. In these exploratory analyses, patients with very high tumor HER2 content (log H2T $\geq 2.1$) did not benefit from adjuvant trastuzumab [HR = 1.23; 95% confidence interval (CI) 0.33–4.62; $P = 0.75$; Figure 5A], whereas patients with HER2-positive disease by CISH with moderately high tumor H2T content (log $< 2.1$) derived significant benefit from trastuzumab (HR = 0.52; 0.28–1.00; $P = 0.050$; Figure 5B). In the subset of patients treated with adjuvant trastuzumab ($n = 100$), patients with very high tumor HER2 content (log H2T $\geq 2.1$) had inferior distant RFS compared with those with lower tumor H2T content (log H2T $< 2.1$) (HR = 2.83; 1.02–7.82; $P = 0.046$).

**discussion**

Identification of HER2 as a therapeutic target represents a major advance in the rational selection of treatment for breast cancer patients. None the less, not all patients whose cancer overexpresses HER2 respond to anti-HER2 therapy [19, 20]. Potential explanations for this include inaccuracy in the testing modalities currently employed to correctly identify HER2-positive tumors, alterations in functional interactions between HER2 and other HER-family proteins that are not detectable with current tests (such as HER2–HER3 heterodimers), alternate forms of HER2 that are resistant to trastuzumab and cannot be distinguished from full-length HER2 using current readily available technologies (e.g. the p95 C-terminal fragment of HER2 generated either by sheddase-dependent cleavage of the HER2 extracellular domain or by alternative translation initiation of the HER2 messenger RNA), loss of phosphatase and tensin homolog or phosphatidylinositol 3-kinase function [21], heterogeneity of HER2 expression, or perhaps other as yet undiscovered biological mechanisms.

The present results, obtained using a novel quantitative measure of HER2 protein expression, suggest that patients with the highest tumor levels of HER2 may gain little benefit from treatment with trastuzumab administered concomitantly for 9 weeks with docetaxel or vinorelbine, whereas patients who have HER2-positive cancer with moderate HER2 levels do benefit. The initiative for carrying out the present analyses came from findings in a cohort of trastuzumab-treated patients diagnosed with advanced breast cancer, where we observed that patients who had the highest primary tumor HER2 levels had similar progression-free survival as women whose cancer was FISH negative by central testing and expressed the lowest levels of HER2 in the cohort [8]. As in the present study, STEPP analyses suggested that the association between H2T and progression-free survival was nonlinear. When the optimal cut-off value, defined by the positional scanning analyses, was used, 16% of the patients with advanced HER2-positive cancer were included in the high H2T category, which is a similar proportion to the 13% found in the present series. These results, although consistent, need to be viewed with caution. When high tumor H2T is examined for correlation with distant RFS in a Cox model that includes trastuzumab treatment and H2T as a categorical variable with log 2.1 as the cut-off point in the present series, the interaction between the treatment and...
log H2T remains nonsignificant \((P = 0.27)\), possibly due to the small size of the high H2T subgroup \((n = 25)\).

These results were obtained using a novel quantitative method that measures tumor HER2 protein levels, but studies where HER2 copy numbers were assessed by FISH may be in line with these findings. An analysis of the Herceptin Adjuvant trial suggests that adjuvant trastuzumab may confer less benefit for patients with the highest ratios of HER2 copy number to chromosome 17 centromere ratios \((HER2/CEP17 > 8; HR = 0.91; 95\% CI 0.60–1.37)\), and there appeared to be little benefit from trastuzumab in the subgroup of patients whose cancer contained the highest copy numbers of HER2 \((HER2/CEP17 > 18; HR = 0.90; 95\% CI 0.60–1.34)\) [3]. A recent analysis of the N9831 phase III trial that also addressed adjuvant trastuzumab did not detect significant benefit from trastuzumab when the HER2/CEP17 ratio was very high \((\geq 15)\), but this subgroup was small in size \((4\%)\) and the finding was not statistically significant [22]. Thus, there may now be evidence from separate cohorts of breast cancer patients treated with various chemotherapy regimens and with different durations of adjuvant trastuzumab administration suggesting that patients with HER2-positive breast cancer with the highest levels of HER2 benefit less from trastuzumab compared with women whose HER2-positive cancer contains moderate levels of HER2. Interestingly, a recent analysis of tumor HER2 content in a large series of breast cancer patients treated with doxorubicin- and cyclophosphamide-containing adjuvant chemotherapy before the trastuzumab era suggests that both high and low HER2-expressing breast cancers are associated with unfavorable outcomes [23].

The biological explanations for this observation remain unknown. When very high tumor HER2 levels are present, the density of HER2 on cancer cells may be so high that the amount of trastuzumab available in the tumor is insufficient to antagonize HER2 signaling completely. HER2 expressed at extreme concentrations might cluster on the cell surface...
causing steric hindrance that impairs access of trastuzumab to its juxtamembrane epitope target on the HER2 extracellular domain. HER2 might undergo a conformational change resulting in cleavage of the extracellular domain resulting in p95 with no binding site for trastuzumab, or have an increased propensity to form heterodimers that are not optimally susceptible to trastuzumab antagonism.

We conclude that quantitative analysis of tumor HER2 protein content is strongly associated with results obtained with CISH. Breast cancer patients who have tumor with very high HER2 content may benefit less from adjuvant chemotherapy plus trastuzumab compared with women whose HER2-positive cancer contains more moderate HER2 levels. Although findings from two other studies that have addressed trastuzumab as first-line therapy for breast cancer patients with very high HER2 expression levels and possible resistance to trastuzumab are in line with the present observations [3, 8], this result needs to be interpreted with caution since it is based on retrospective exploratory analyses and a relatively small number of events. Optimal management of patients with very high H2T levels might include larger than the standard doses of trastuzumab, novel anti-HER2 agents, or combinations of HER2-targeting agents, which remains to be investigated.

**acknowledgements**

We are grateful for FinHer trial participants, investigators, study nurses and financial supporters. Presented in part at the 31st San Antonio Breast Cancer Symposium, San Antonio, TX, 10–14 December 2009.

**funding**

Monogram Biosciences, Inc., South San Francisco; Academy of Finland; Helsinki University Central Hospital Research Funds (TYH2009304); Cancer Society of Finland; Sigrid Juselius Foundation.

**disclosure**

HJ and P-LK-L: honoraria from Sanofi-Aventis, Roche; JS, WH, JW, AP, YL and MB: employees of Monogram Biosciences Inc.; PB: honoraria from Roche; SJ: honoraria from Roche, GlaxoSmithKline and Novartis and other remuneration from Amgen; P-LK-L: research funding from Roche, Sanofi-Aventis and Pierre-Fabre; ML, VK, RK, TT-H and JI declare no conflicts of interest.

**references**

12. Slamon D, Eiermann W, Robert N et al. Phase III randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel (AC→T) with doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab (AC→TH) with docetaxel, carboplatin and trastuzumab (TCH) in Her2neu positive


