**HER2 Status in Advanced or Metastatic Gastric, Esophageal, or Gastroesophageal Adenocarcinoma for Entry to the TRIO-013/LOGiC Trial of Lapatinib**

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**Abstract**

HER2/ERBB2 status is used to select patients for HER2-targeted therapy. HER2/ERBB2 amplification/overexpression of upper gastrointestinal (UGI) adenocarcinomas was determined locally or in two central laboratories to select patients for the TRIO-013/LOGiC trial of chemotherapy with or without lapatinib. Patients selected locally had central laboratory confirmation of HER2 amplification for inclusion in the primary efficacy population. HER2 was assessed with PathVysion or IQ PharmDx FISH and HercepTest immunohistochemistry assays. Associations with outcomes were retrospectively evaluated. Overall, HER2 status was determined in UGI cancers from 4,674 patients in a central laboratory for eligibility (1,995 cases) and for confirmation of local HER2 results (333 cases). Of 1,995 adenocarcinomas screened centrally, 322 (16.1%) had HER2-amplified disease with 29 (1.5%) showing HER2 genomic heterogeneity. Men and older patients had higher rates of amplification. Of 545 patients accrued to the trial (gastric, 87.3%; GEJ, 8.3% and esophageal cancer, 4.4%), 487 patients (89%) were centrally confirmed as having HER2-amplified disease. Concordance between central and local HER2 testing was 83%. Concordance between PathVysion and IQ PharmDx FISH assays was 99% and FISH in the two central laboratories was 95%. Lapatinib-treated Asian participants and those less than 60 years had significant improvement in progression-free survival (PFS), particularly among those whose cancers had 5.01–10.0-fold amplification of HER2. In conclusion, HER2 is commonly amplified in UGI adenocarcinomas with amplification highly correlated to overexpression, and HER2 amplification levels correlated with PFS. While HER2 genomic heterogeneity occurs, its prevalence is low.

**Introduction**

The human epidermal growth factor receptor (EGFR) type 2 (HER2) gene is commonly amplified (1–3) and overexpressed in adenocarcinomas of the stomach, gastroesophageal junction (GEJ), and esophagus and is a potential target for therapeutic intervention. Although the role of HER2 as a prognostic marker in gastric cancer remains an issue of debate, HER2 amplification/overexpression is an important therapeutic target for trastuzumab and is associated with significant improvements in progression-free (PFS) and overall survival (OS; ref. 4). Routine diagnostic testing is now recommended in gastric and GEJ adenocarcinomas based on these findings that led to approval of the drug by the FDA (5) and European Medicines Agency (EMA).
Here we report on assessments of HER2 status conducted for patient entry to a trial of lapatinib, a dual tyrosine kinase inhibitor of HER2 and EGFR, in combination with chemotherapy in patients with advanced HER2-positive upper gastrointestinal (UGI) adenocarcinomas. The TRIO-013/LOGiC study was based on the efficacy of lapatinib in patients with HER2-positive breast cancer (6–9) and activity both in vitro and in vivo with UGI cancer cell lines exhibiting HER2 gene amplification (10). In addition, modest single-agent activity had been observed in patients with UGI cancers. At the time this trial was initiated, no anti-HER2 agent had demonstrated activity in a systematic study of this disease. This retrospective study of HER2 gene amplification and overexpression in UGI adenocarcinomas was initiated to explore the potential roles of HER2 amplification levels and HER2 genomic heterogeneity in lapatinib treatment responsiveness, as measured by improved PFS or improved OS in the LOGiC clinical trial.

Materials and Methods

TRIO-013/LOGiC clinical trial

The TRIO-013/LOGiC clinical trial [Lapatinib optimization study in ErbB2 (HER2) positive gastric cancer: a phase III global, blinded study designed to evaluate clinical endpoints and safety of chemotherapy plus lapatinib] was a double-blind, randomized, multi-center, phase III study (ClinicalTrials.gov: NCT00680901) of capecitabine and oxaliplatin (CapeOx) without or with lapatinib conducted in patients with metastatic HER2-positive gastric, GEJ, and esophageal adenocarcinoma between June 2008 and January 2012 at 186 centers in 22 countries. The details of patient accrual, treatment, toxicities, and outcomes are described separately (11). Here we describe HER2 testing issues and their associations with outcomes. The study was conducted in accordance with the current ethical principles outlined in the Declaration of Helsinki guidelines (12).

HER2 status for enrollment in the TRIO-013/LOGiC clinical trial

Patients enrolled in this trial had advanced, HER2-positive UGI adenocarcinomas as evaluated in local laboratories (IHC 2+, or FISH-amplified, or IHC3+, or FISH-amplified) or in a central laboratory (FISH-amplified). Patients enrolled in the trial with a positive HER2 local test result were required to have tumor tissue submitted to one of the two central laboratories for the assessment of HER2 gene amplification by the HER2 PathVysion FISH assay (Abbott Molecular, Inc.) and expression by IHC (HercepTest, Dako Biotechnology) for confirmation of the local result. Although patients could be enrolled to the trial based on HER2-status in a local laboratory, patient entry to the trial (11) were stratified according to history of prior adjuvant or neoadjuvant therapy and region (Asia, North America, and rest of the world) then randomized to receive oxaliplatin (130 mg/m² on day one) and capecitabine (850 mg/m², twice a day, days 1–14) either with lapatinib (1,250 mg daily, day 1–21) or with placebo (daily, day 1–21).

Identification of PEP

As no HER2 tests were approved for use in UGI cancers at the start of the trial, Translational Research In Oncology (TRIO)/Cancer International Research Group (CIRG) selected the PathVysion dual-color FISH assay (HER2 PathVysion FISH assay, Abbott Molecular, Inc.) as the primary method for assessment of HER2 amplification in patients' cancers to establish a consistent standard for HER2 positivity across the trial based on previous approval as a companion diagnostic for breast cancer patient selection to HER2-targeted therapies and experience with this assay method (8, 13–18). Formalin-fixed paraffin-embedded (FFPE) tissues or unstained sections were submitted to a central laboratory for the assessment of HER2 status (Fig. 1A).

Laboratory assessment of HER2 status

HER2 amplification was determined by FISH, as described (refs. 15–17; Supplementary Data), in one of two central laboratories, one at the University of Southern California (Los Angeles, CA) and the other at the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). The entire processed tissue section was routinely screened for focal areas of carcinoma with HER2 status that differed from the remaining tumor both by IHC and FISH (HER2 genomic heterogeneity). When such areas of disparate HER2 gene copy number were identified they were scored separately by FISH and the tumor area estimated. HER2 amplification was defined as a HER2 gene-to-CEP17 ratio of 2.0 or greater, the FDA-approved ratio; an average HER2 gene copy number of 4.0 or greater was not required (19–21). Similarly for IHC, high geographic variability in HER2 immunostaining (e.g., areas of IHC 3+ and other areas of IHC 0/+ that are consistent with HER2 genomic heterogeneity) were noted and the percent of each area was estimated (see Fig. 2 and Supplementary Figs. S1 and S2).

HercepTest IHC Assay

During the conduct of this trial it became apparent, based on reports from ToGA trial investigators (4, 22), that there may be some controversy related to HER2 testing in gastric and GEJ adenocarcinomas. Therefore, TRIO/CIRG investigators and the trial sponsor decided to also implement IHC testing for HER2 protein in the central laboratories. The HercepTest (Dako, Inc.), a companion diagnostic initially approved for breast cancer assessments and subsequently, approved for gastric and GEJ adenocarcinomas, was used to determine HER2 protein expression as described previously (15–17) and summarized in Supplementary Methods.

Concordance study

The HER2 PathVysion FISH assay was selected as the primary testing method for assessment of HER2 status, however, during the trial, questions were raised about assay comparability with the HER2 IQFISH pharmDx method (Dako Corporation) which became FDA-approved for HER2 testing in gastric cancer for selection of patients to trastuzumab therapy. To assess
agreement rates between assay methods and agreement rates in the two central laboratories, a separate cohort of 488 UGI adenocarcinoma cases, 419 (86%) gastric, 43 (8.8%) gastro-esophageal junction (GEJ), and 26 (5.3%) esophageal cancers, were procured from commercial providers (Asterand Bioscience, Analytical Biological Services, Inc., Proteogenex, Inc., Individumed GmbH) as FFPE blocks and screened by HercepTest to ensure representation of all four IHC staining intensities (Fig. 1B). All IHC3+ and IHC2+ cases were included in the concordance study. Whereas IHC1+ and IHC 0 cases were grouped by primary tumor site (gastric, GEJ, and esophageal) with inclusion of all GEJ and esophageal carcinomas, then gastric carcinomas were randomly selected. The 159 selected cases were masked and sent to two central laboratories for HER2 FISH testing, masked to all information.

Ethics committee approval
Ethics committees and/or institutional research boards at each clinical site and at each central laboratory approved the study and the research protocols. All study participants signed an informed consent to enroll in the study.

Statistical analyses
Statistical aspects of the LOGIC study design and statistical methods for analyses are summarized elsewhere (11). Briefly, we performed a Cox proportional hazards model on treatment for each of the subgroups to compute a HR and 95% confidence interval. Then using the unstratified log-rank test, we computed P values, as well as median survival times and 95% confidence intervals for both PFS and OS. Although the analyses between FISH results and outcomes reported here are exploratory and
hypothesis generating, the differences observed retrospectively in a clinical trial were still considered in our analyses as potentially meaningful, namely, they warrant further exploration in a subsequent study, or not. We chose a $P$ value of $\leq 0.05$ as our threshold to suggest a marker/analysis was meaningful. For concordance analyses, positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) with 95% confidence intervals were determined as described previously (23) and summarized in Supplementary Methods.

Results

HER2 testing in the TRIO-013/LOGiC trial

Overall, 4,674 patients were registered in the central database and underwent eligibility screening for the TRIO-013/LOGiC trial (Fig. 1A). Central FISH testing was performed in 1995 patients for eligibility and 333 for confirmation of a local HER2 result; one patient enrolled and deemed HER2 positive by a local laboratory did not have tumor tissue remaining for central confirmation of
HER2 status and FISH results were not obtained from six additional samples (Fig. 1A). Of the 1,995 carcinomas screened by a central laboratory for trial enrollment, 322 (16.1%) had HER2 amplification (Fig. 1A). 29 (1.5%) of which showed HER2 genomic heterogeneity (Fig. 2). The adenocarcinomas with genomic heterogeneity represent 9% of identified HER2-amplified cases primarily screened in the central laboratories. An additional 33 cases with HER2 genomic heterogeneity were identified among those screened in local laboratories and confirmed in a central laboratory. Demographic data for these patients demonstrates a higher frequency of disease among men and shows that men and older patients have higher rates of HER2 gene amplification (Table 1).

In patients whose cancers were centrally assessed, either for primary assessment or confirmation of local laboratory results, HER2 status by both FISH and IHC is known for 1,250 with 457 of (83.5%) amplification status by both FISH and IHC is known for 1,250 with 457 of 547 (83.5%) ampliﬁed cases IHC2+ or IHCh 3+ and 680 of 703 (96.7%) non-ampliﬁed cases either IHCh 1+ or IHCo (Table 2). The overall concordance rate between FISH and IHC results for HER2 status was 91% [95% conﬁdence interval (CI): 89.2%–92.4%.] In our dataset, there were no cases that were IHC3+/FISH-negative. The majority (84%) of IHC2+ cases were HER2 amplified, while relatively few IHCo (n = 33, 6%) or IHCh1+ (n = 57, 26%) cancers were HER2 amplified (Table 2). The distribution across the various IHC immunostaining categories progressively transitions from predominantly IHCo (515/703, 73.3%) without amplification (FISH ratio <2.0), to a more even distribution [IHCo (18%), IHCh 1+ (28%), IHCh 2+ (35%), IHCh 1+ (19%) with low-level amplification (FISH ratio of 2.0–5.0) to progressively higher distributions of stronger IHC staining [IHCo (2%), IHCh 1+ (6%), IHCh 2+ (24%), IHCh 3+ (67%)] for FISH ratios >5.0–10.0 and the highest distribution [IHCh 3+ (91%)] for FISH ratios >10.0 (Table 2).

Of 333 patients with trial enrollment based on local laboratory testing, 332 had tumor tissue available for subsequent reassessment in a central laboratory (Supplementary Table S1). Concordance between central and local HER2 testing was 84.4% [275/326, Fig. 1A]. As samples were not required for re-evaluation in the central laboratory for patients not enrolled to the trial, overall concordance in this population could not be assessed.

The agreement rate between local and central laboratory HER2 testing was dependent on the HER2 testing method used in the local laboratory and concordance varied accordingly. Among the 332 cancers evaluated both locally and centrally, 259 cases were tested in local laboratories by ISH alone, 33 by ISH and IHC, and 40 by IHC alone. Among the 292 cases tested locally by ISH, the central laboratory determined the HER2 gene amplification status in 286 of the cases. HER2-amplified status was confirmed for 250; however, 36 were assessed as not amplified for an ISH

### Table 1. Demographic information for 1995 patients whose cancers were assessed initially for HER2 status in the central laboratories for eligibility

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HER2-amplified</th>
<th>HER2 not ampliﬁed</th>
<th>IHCGene ampliﬁcation</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;39 (n = 92)</td>
<td>11 (11.9%)</td>
<td>80 (87%)</td>
<td>1 (1%)</td>
<td>92</td>
<td>0.0038</td>
</tr>
<tr>
<td>40–49 (n = 224)</td>
<td>29 (12.9%)</td>
<td>192 (85.8%)</td>
<td>3 (1.3%)</td>
<td>224</td>
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</tr>
<tr>
<td>50–59 (n = 529)</td>
<td>75 (14.2%)</td>
<td>449 (84.9%)</td>
<td>5 (0.9%)</td>
<td>529</td>
<td></td>
</tr>
<tr>
<td>60–69 (n = 585)</td>
<td>105 (17.9%)</td>
<td>471 (80.6%)</td>
<td>9 (1.5%)</td>
<td>585</td>
<td></td>
</tr>
<tr>
<td>70–79 (n = 320)</td>
<td>62 (19.7%)</td>
<td>246 (76.9%)</td>
<td>11 (3.4%)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>≥80 (n = 27)</td>
<td>9 (33%)</td>
<td>18 (67.0%)</td>
<td>0 (0%)</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>NA (n = 30)</td>
<td>1</td>
<td>29</td>
<td>0</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>293 (16.2%)</td>
<td>1,485 (82.2%)</td>
<td>29 (1.6%)</td>
<td>1,807</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 1,207)</td>
<td>211 (17.5%)</td>
<td>974 (80.7%)</td>
<td>22 (1.8%)</td>
<td>1,207</td>
<td>0.0262</td>
</tr>
<tr>
<td>Female (n = 592)</td>
<td>82 (13.9%)</td>
<td>503 (85%)</td>
<td>7 (1.1%)</td>
<td>592</td>
<td></td>
</tr>
<tr>
<td>NA (n = 8)</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>293 (16.2%)</td>
<td>1,485 (82.2%)</td>
<td>29 (1.6%)</td>
<td>1,807</td>
<td></td>
</tr>
<tr>
<td>Country of origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brazil</td>
<td>41 (18.1%)</td>
<td>177 (78.0%)</td>
<td>5 (3.5%)</td>
<td>227</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>8 (15.6%)</td>
<td>50 (84.7%)</td>
<td>1 (1.7%)</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Chile</td>
<td>22 (96.2%)</td>
<td>113 (83.1%)</td>
<td>1 (0.7%)</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>Estonia</td>
<td>8 (14.3%)</td>
<td>48 (85.7%)</td>
<td>0</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Hong Kong</td>
<td>4 (22.2%)</td>
<td>14 (77.8%)</td>
<td>0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Hungary</td>
<td>9 (15.6%)</td>
<td>56 (84.8%)</td>
<td>1 (1.5%)</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Israel</td>
<td>5 (13.2%)</td>
<td>33 (86.8%)</td>
<td>0</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>23 (20.7%)</td>
<td>87 (78.4%)</td>
<td>1 (0.9%)</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>Korea</td>
<td>2 (18.2%)</td>
<td>9 (81.8%)</td>
<td>0</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Mexico</td>
<td>4 (9.5%)</td>
<td>37 (88.1%)</td>
<td>1 (2.4%)</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Netherlands</td>
<td>4 (28.6%)</td>
<td>7 (70.0%)</td>
<td>3 (21.4%)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Peru</td>
<td>4 (21.0%)</td>
<td>14 (73.0%)</td>
<td>1 (5.3%)</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Poland</td>
<td>52 (17.1%)</td>
<td>250 (82.2%)</td>
<td>2 (0.7%)</td>
<td>304</td>
<td></td>
</tr>
<tr>
<td>Russia</td>
<td>29 (20.6%)</td>
<td>111 (78.7%)</td>
<td>1 (0.7%)</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Taiwan</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Thailand</td>
<td>1 (4.5%)</td>
<td>19 (86.4%)</td>
<td>2 (9.1%)</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>3 (14.3%)</td>
<td>18 (85.7%)</td>
<td>0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>United States</td>
<td>11 (23.4%)</td>
<td>35 (74.5%)</td>
<td>1 (2.1%)</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Ukraine</td>
<td>62 (13.1%)</td>
<td>406 (85.7%)</td>
<td>6 (1.2%)</td>
<td>474</td>
<td></td>
</tr>
</tbody>
</table>

aOf 1,995 cases centrally assessed for HER2 status, a FISH result was not available for analysis in 188 of the cases.

bP was determined using χ² statistical test. It should also be noted that many Asian sites used a local laboratory, for example, in China, and provided tissue sections in the central laboratory only for confirmation of their status (primary efficacy population) after accrual to the trial (see Fig. 1A). NA, not available.
Table 2. TRIO-013/LOGiC Clinical Trial: comparison of HER2 testing by FISH and IHC in two central laboratories

<table>
<thead>
<tr>
<th>PathVysion FISH assay</th>
<th>HER2 not ampl</th>
<th>HER2 ampli*</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC 0</td>
<td>515 (94%)</td>
<td>33 (6%)</td>
<td>548</td>
</tr>
<tr>
<td>IHC 1</td>
<td>165 (74.3%)</td>
<td>57 (25.7%)</td>
<td>222</td>
</tr>
<tr>
<td>IHC 2</td>
<td>23 (16%)</td>
<td>121 (84%)</td>
<td>144</td>
</tr>
<tr>
<td>IHC 3</td>
<td>0</td>
<td>336 (100%)</td>
<td>336</td>
</tr>
<tr>
<td>Total</td>
<td>703</td>
<td>547</td>
<td>1,250</td>
</tr>
</tbody>
</table>

PathVysion FISH assay

<table>
<thead>
<tr>
<th>FISH Ratios</th>
<th>IHC 0</th>
<th>IHC 1</th>
<th>IHC 2</th>
<th>IHC 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10.0, n (%)</td>
<td>515</td>
<td>164</td>
<td>24</td>
<td>0</td>
<td>703</td>
</tr>
<tr>
<td>2.0–5.0, n (%)</td>
<td>29</td>
<td>45</td>
<td>57</td>
<td>31</td>
<td>162</td>
</tr>
<tr>
<td>&gt;5.0–10.0, n (%)</td>
<td>4</td>
<td>12</td>
<td>45</td>
<td>124</td>
<td>185</td>
</tr>
<tr>
<td>&gt;10.0, n (%)</td>
<td>0</td>
<td>1</td>
<td>18</td>
<td>181</td>
<td>200</td>
</tr>
<tr>
<td>Total</td>
<td>548</td>
<td>181</td>
<td>144</td>
<td>336</td>
<td>1,250</td>
</tr>
</tbody>
</table>

NOTE: Of the 547 HER2-amplified UGI adenocarcinomas, 90 (16.5%) had only IHC 0 or 1+ immunostaining for HER2 protein. Overall agreement between FISH and IHC results for HER2 status was 91% (1,137/1,250; 95% CI: 89.2–92.4%). Other investigators have reported lower concordance rates between IHC and FISH, especially in the IHC 2+ group where less than 40% have been HER2 amplified by FISH, in esophageal/GEJ or gastric adenocarcinomas (3, 52).

Overall concordance between LOGiC central laboratories for 151 cases successfully tested by PathVysion HER2 FISH assay in both laboratories was 94.7% (95% CI: 89.9–97.3%) with 93.8% PPA (95% CI: 85.2–97.9%) and 95.1% NPV (95% CI: 89.1–97.9%; Supplementary Table S5). Discordance between the two central laboratories was primarily related to differences in scoring of 14 samples with highly variable HER2 immunostaining, consistent with HER2 genomic heterogeneity (Supplementary Results, Supplementary Figs. S1 and S2).

Concordance study for different HER2 FISH assay methods between the two central laboratories

Before accrual to the LOGiC trial was complete, the ToGA trial was completed and the FDA, subsequently, approved two companion diagnostic assays, the Dako HercepTest IHC assay (October, 2010) and the Dako HER2 IIFISH pharmDx FISH assay (February, 2013) for selection of gastric and GEJ cancer patients to trastuzumab plus chemotherapy (4). Therefore, we performed a concordance study to demonstrate agreement between the PathVysion HER2 FISH assay and the Dako HER2 IIFISH pharmDx HER2 assay (Fig. 1B and Supplementary Results and Supplementary Tables S4 and S5). UGI adenocarcinomas from 488 patients who were not enrolled in the TRIO-013/LOGiC trial were used for the concordance studies (Fig. 1B). To ensure the full spectrum of HER2 expression was more evenly represented in the concordance comparison rather than a predominance of HER2 low-expression, not-amplified cases, the tumors were initially processed and scored by IHC with the selection of 159 cases for further analysis by FISH. The central laboratory was blinded to the prior IHC status during FISH analyses performed in the central laboratories. The initial IHC results from all 488 UGI cancers showed highly variable immunostaining in 17 cases (3.5%) that was considered consistent with potential HER2 genomic heterogeneity (Supplementary Figs. S1 and S2). The central masking/randomization center included 14 of these IHC-variable cases that had sufficient tumor for inclusion in the concordance study thus increasing the rate of potential “HER2 heterogeneity” from 3.5% (17/488) to 8.8% (14/159). Of the 159 cases selected for inclusion in the concordance study: 31 exhibited HER2 staining indicative of IHC3+, 20 IHC2+, 55 IHC1+, and 53 IHC0 (Fig. 1B).

Overall concordance between LOGiC central laboratories for 151 cases successfully tested by PathVysion HER2 FISH assay in both laboratories was 94.7% (95% CI: 89.9–97.3%) with 93.8% PPA (95% CI: 85.2–97.9%) and 95.1% NPV (95% CI: 89.1–97.9%; Supplementary Table S5). Discordance between the two central laboratories was primarily related to differences in scoring of 14 samples with highly variable HER2 immunostaining, consistent with HER2 genomic heterogeneity (Supplementary Results, Supplementary Figs. S1 and S2).

Summary of clinical outcomes in the TRIO-013/LOGiC trial

In total, among the 545 patients accrued to the trial, 487 patients had central laboratory–confirmed HER2-amplified disease to comprise the primary efficacy population (PEP); while 51 had HER2 nonamplified disease and HER2 amplification status was not determined in 7 patients (Table 3 and Fig. 1A).

Among the 487 patients with HER2-amplified cancers in the PEP, 424 (87.1%) had gastric, 43 (8.8%) GEJ, and 20 (4.1%) esophageal cancers. In the PEP, 297 had cancers that were HER2 IHC3+ [61%], 108 were IHC2+ [22%], 54 were IHC1+ [11%], 27 were IHC0 (6%), and one was IHC unknown. As reported separately in detail (11), the LOGiC trial did not meet its primary endpoint of a significant improvement in OS among those patients randomized to receive chemotherapy with lapatinib [HR = 0.91 (95% CI: 0.73–1.12), P = 0.35]; however, a significant improvement in PFS was observed [HR = 0.82 (95% CI: 0.68–1.00), P = 0.038]. Subset analyses demonstrated meaningful improvements in both PFS and OS for selected subsets of patients, such as those accrued in Asian countries and those patients less than 60 years of age at trial enrollment (Table 3 and Supplementary Table S6). Among Asian participants and those less than 60 years of age, we observed greater PFS related to higher levels of HER2 gene amplification (Table 3). In the overall trial population (>10.0-fold) as well as among the Asian population (≥3.01–10.0 and >10.0-fold) and in the population less than 60 years of age the HR for PFS became more pronounced only at higher levels of HER2 gene amplification (>5.0–10.0 and >10.0 fold; Table 3). HER2 ratio was not associated with OS in the overall population or in the subpopulations analyzed for PFS (Table 3; P > 0.05), with...
the exception of Asian participants whose cancers had 5.01–10.0 HER2 gene amplification levels.

Among the 81 cases included in the trial with HER2 gene amplification by FISH with only IHC0/1þ for HER2 protein, HRs for PFS were 1.12 (P = 0.65) and OS 0.91 (P = 0.71). In contrast, those patients whose HER2-amplified UGI cancers were also IHC2þ/3þ had greater improvement in PFS [HR = 0.76; (95% CI: 0.62–0.94), P = 0.0096] but not OS [HR = 0.86 (95% CI: 0.68–1.09), P = 0.21]. Among the 148 participants from Asian countries with HER2-amplified/IHC2þ/3þ UGI adenocarcinoma, PFS (HR = 0.59; P = 0.0020) and OS (HR = 0.60, P = 0.0108) were greatly improved by the addition of lapatinib to chemotherapy. These results were highly intriguing and will be proposed for further prospective assessments in subsequent studies.

**Discussion**

Gastric cancer remains the third leading cause of cancer-related mortality in the world (24). Treatment typically involves gastric resection; however, surgery alone is generally only curative for patients with early-stage disease. For patients with advanced resectable disease, survival rates remain poor but are improved by chemotherapy and possibly radiotherapy (25–27).

HER2 amplification/overexpression as a prognostic marker

The majority of gastric cancer studies do not show an association of HER2 status with clinical outcomes in the absence of chemotherapy and/or radiotherapy (28–37); however, studies of patients treated with chemotheraphy and/or radiotherapy demonstrate a survival advantage for patients whose gastric cancers lack HER2 gene amplification/overexpression (32, 38–41). An additional large study of patients with adenocarcinomas of the gastric cardia, GEJ, and esophagus treated by surgical resection (1980–1997) showed no difference in either disease-specific survival or OS among patients whose cancers had HER2 gene amplification (42). The potential role of HER2 as a predictive marker of responsiveness to chemotherapy and radiotherapy is supported by a retrospective analysis of HER2 gene amplification and overexpression in gastric cancers from patients in the INT-0116/SWOG9008 phase III gastric cancer clinical trial comparing surgery alone with surgery and chemotherapy (5-fluorouracil)/radiotherapy (13). Patients whose gastric cancers were HER2-negative and were treated with chemoradiation therapy had a longer disease-free and overall survival than similar patients treated with gastrectomy alone. In contrast, no benefit from chemotherapy and radiotherapy was observed among the subset of patients whose gastric cancers had HER2 amplification/overexpression (13).

HER2 amplification/overexpression as a target for therapy

HER2 amplification/overexpression is an important therapeutic target for trastuzumab in metastatic gastric and gastroesophageal cancer (4), as it is in metastatic breast cancer (9, 18, 43). Both the Trastuzumab for Gastric Cancer (ToGA) and LOGiC trials were...
initiated because of medical need in gastric/gastroesophageal/esophageal carcinoma, a high HER2 positivity rate and an already established toxicity profile in breast cancer patients. The ToGA clinical trial assessed efficacy and safety of trastuzumab added to capecitabine or fluorouracil and cisplatin chemotherapy for first-line treatment of advanced gastric or GEJ cancers with overexpression of HER2 (4). Among patients assigned to chemotherapy with trastuzumab in the ToGA trial, median OS was 13.8 months (95% CI, 12–16) compared with 11.1 months for those assigned to chemotherapy alone (HR = 0.74; 95%CI: 0.60–0.91; P = 0.0046). In the LOGIC trial, the median OS was 12.2 months (95% CI: 10.6–14.2) for patients assigned to capecitabine and oxaliplatin chemotherapy with lapatinib compared with 10.5 months (95% CI: 9.0–11.3) for patients assigned to chemotherapy and oxaliplatin chemotherapy with lapatinib. Although differences in survival among the overall LOGIC trial population were not observed, Asian patients and patients under 60 years of age did show noticeable improvements in OS (11). However, we did not find any systematic difference in HER2 status by either IHC or FISH among these subgroups that could account for the differences in outcome.

HER2 testing in UGI cancers

Routine diagnostic HER2 testing is recommended in gastric and GEJ adenocarcinomas based on findings from the ToGA trial that led to regulatory approval of trastuzumab for this indication in 2010. We found a high concordance rate for HER2 gene amplification status determined with two different FISH1 assays (99% agreement) and between two different central laboratories when the same FISH assay is used (95% agreement), even though the comparison samples were enriched for cases with HER2 genomic heterogeneity (8.8%). Agreement between local laboratory and central laboratory for HER2 FISH1 assays was only 87%. Agreement between local laboratory and central laboratory for HER2 IHC assays was less (58%) (Supplementary Table S3); however, the number of cases available for this latter analysis was quite limited. Overall agreement between HER2 IHC and FISH assays in the central laboratory was approximately 90%, 91% in the LOGIC clinical trial cases (n = 1,250), and 88% in the concordance cohort cases (n = 155). The discrepancies identified for HER2 status determined by IHC compared with FISH, as well as disagreements between local and central laboratory testing, have the potential to inappropriately select some patients for HER2-targeted therapy as well as the potential to inappropriately deny other patients such treatment. Our findings suggest this is of particular concern when IHC is used as the primary test, as addressed elsewhere in detail (20).

There has been some concern that UGI endoscopic biopsies may not provide sufficient tissue for adequate identification of all cancers with this alteration. Although we did not have both endoscopic biopsy samples and resection specimens from the same patients for a direct case-by-case comparison, our HER2 amplification rates among patients with endoscopic biopsy specimens (18.3%) compared with patients who had surgical resection specimens (15.0%) suggests that endoscopic biopsies are likely to be representative of the HER2 amplification status (Supplementary Results and Supplementary Table S7).

HER2 genomic heterogeneity

HER2 genomic heterogeneity was observed overall in approximately 1%–2% of the UGI adenocarcinomas in this study (Table 1, Fig. 2 and Supplementary Figs. S1 and S2). The rate did not vary significantly by patient age, gender, country, or anatomical site. Although the total number of these cases identified by the central laboratory was modest (n = 62), 51 of the cases were identified in patients who were subsequently randomly assigned to study treatment, 24 to the lapatinib treatment arm and 27 to the control arm, no significant differences were observed in PFS or OS. It should be noted that our definition of HER2 genomic heterogeneity differs from the definition used in CAP guidelines (44). The definition of HER2 genomic heterogeneity used in the LOGIC trial was the same as used for trials of breast cancer screened in the same two TRIO/BCIRG central laboratories (16) for the BCIRG-005 (45), BCIRG-006 (6) and BCIRG-007 (46) trials. We consider HER2 genomic heterogeneity to exist when at least one geographically defined area of contiguous tumor cells are identified within a cancer that differs from the remaining tumor cells by HER2 amplification status (i.e., HER2-amplified versus HER2 not amplified). This differs from the definition established by the CAP guidelines for breast cancer (44). The CAP criteria do not distinguish or require a geographically defined area but simply that the scores of 20 or more cells have at least 5% of scored cells with a HER2-to-CEP17 ratio that is ≥ 2.2 and less than 50% of the tumor cells have such a ratio. The guidelines state "HER2 genetic heterogeneity (GH) exists if there are more than 5% but less than 50% of infiltrating tumor cells with a ratio higher than 2.2." We also differ from the guidelines in our approach to scoring HER2 heterogeneity. According to the guidelines, "if 20 cells are counted and at least one cell is identified with a HER2/CEP17 signal ratio higher than 2.2, this specimen contains GH. Likewise, if 60 cells are examined and 3 or more cells have a ratio higher than 2.2, HER2 GH is present." Using this definition leads to substantially higher rates of HER2 heterogeneity by FISH ranging from 14% to 26% in breast cancers (47, 48). However, use of the same definition in gastric, GEJ, and esophagus adenocarcinomas demonstrated only 20 of 675 (2.9%) have HER2 genetic heterogeneity (42). These studies contrast with our previously reported lower rates (0.5% and 1.1%) in breast cancer (15, 16, 21) and similarly low rate for UGI cancers reported here. The CAP definition (44) may lead to the inclusion of a number of cases that do not contain any HER2 gene amplification even in a portion of the tumor. For example, we would consider some of the results generated by the CAP definition to reflect inclusion of (scattered) cancer cells in which a proportion of the cells (exceeding 5%) are in G2- or M-phase of the cell cycle where DNA has been duplicated leading to doubling of the number of copies of HER2 gene but without a doubling of chromosome 17 centrosomes as the centromeres remain fused until late metaphase. These (scattered) individual cells have a HER2 FISH ratio greater than 2.0, are not amplified, but contribute to "genetic heterogeneity" by the CAP definition. These tumor cells are, likely, simply proliferating (Campeau A and Press MF, unpublished data).

As illustrated (Fig. 2, Supplementary Figs. S1 and S2), we required that the differing geographic tumor areas be scored separately and that one area of contiguous tumor cells have an overall FISH ratio less than 2.0 and the other area have a ratio greater than 2.0 with distinctly aggregated or clustered HER2 gene signals in the amplified cancer cells. Accordingly, 62 cases with HER2 genomic heterogeneity were identified in this study with 51 accrued to the trial. Only two of these cases had genomic
heterogeneity in a subpopulation of tumor cells representing less than 5% of the total area of the tumor. A limitation of our estimates of HER2 genomic heterogeneity is that we seldom had more than a single tissue block from the primary carcinoma from which to make these evaluations. Therefore, our frequencies should be considered from this perspective.

Association of lapatinib responsiveness with HER2 amplification level

In breast cancer clinical trials of lapatinib, we have found a relatively uniform HR for PFS that was independent of HER2 amplification level in HER2-amplified breast cancer patients (15). Only patients whose metastatic disease lacked HER2 amplification (HER2 FISH ratio <2.0) showed no improvement in PFS with lapatinib treatment (HR = 1.09; 95% CI: 0.86–1.37; n = 390), while patients whose metastatic disease had low-level (2.0–5.5-fold) amplification (HR = 0.48; 95% CI: 0.28–0.83; n = 82), moderate (5.5–7.6-fold; HR = 0.35; 95% CI: 0.18–0.69; n = 89), high (7.6–10.1-fold; HR = 0.58; 95% CI: 0.33–1.05; n = 87), or very high-level (>10.1-fold) amplification (HR = 0.42; 95% CI: 0.24–0.74; n = 88) levels all showed improved PFS associated with lapatinib treatment.

In contrast, this trial of lapatinib in UGI cancer patients with advanced disease showed an association between lapatinib treatment and improved PFS only at higher levels of HER2 amplification. In the entire trial population of UGI cancer patients, those without amplification (FISH ratios <2.0; HR = 0.89; 95% CI: 0.50–1.59; n = 51), with low-level amplification (FISH ratios 2.0–5.0; HR = 0.90; 95% CI: 0.63–1.29; n = 140) and with moderate-level amplification (FISH ratios 5.0–10.0; HR = 0.92; 95% CI: 0.66–1.28; n = 171) showed no significant improvement in PFS, while UGI patients whose metastatic cancers had high-level amplification (FISH ratios >10.0; HR = 0.64; 95% CI: 0.47–0.88; n = 176) showed significant improvements in PFS with lapatinib treatment. Similarly, in the trial population of Asian UGI cancer patients, those whose cancers lacked HER2 amplification (FISH ratios <2.0; HR = 0.99; 95% CI: 0.37–2.63; n = 21) as well as those whose cancers had low-level amplification (FISH ratios 2.0–5.0; HR = 0.77; 95% CI: 0.44–1.37; n = 56) showed no significant PFS improvement, while those whose cancers had moderate-level amplification (FISH ratios 5.0–10.0; HR = 0.62; 95% CI: 0.66–1.02; n = 84) and high-level amplification (FISH ratios >10.0; HR = 0.59; 95% CI: 0.33–1.04; n = 53) showed significant improvements in PFS with lapatinib treatment. Similar observations were made among UGI cancer patients less than 60 years of age at trial entry who only patients whose adenocarcinomas with low-level HER2 amplification did not show improvements in PFS with lapatinib treatment.

Other investigators have reported similar associations between improved clinical outcomes and moderate or high levels of HER2 gene amplification in gastric cancer patients treated with trastuzumab (49, 50). In a cohort of 90 patients, Gomez-Martin and colleagues report a mean HER2/CEP17 ratio of 4.7 as the optimal cut-off value discriminating sensitive and refractory patients (P = 0.005); a ratio of 4.45 as the optimal cut-off for survival >12 months (P = 0.005), and a ratio of 5.15 as the optimal cut-off for survival >16 months (P = 0.004; ref. 49). Although there were only 66 patients in the Ock and colleagues study with HER2 gene amplification status determined by FISH, the conclusions were similar (50). Neither of these studies have a control, untreated group to confirm that patients with a HER2/CEP17 ratio less than 4.7 lack significant improvement in outcomes; however, patients whose cancers have ratios more than approximately 5 show improved outcomes compared with those whose cancers have lower levels of gene amplification. These findings are consistent with our own findings of greater benefit among patients whose cancers have higher levels of amplification (ratio 5.0–10 and >10).

The LOGiC trial did not show a significant association with OS (11), while ToGA did (4). LOGiC demonstrated a significant association with PFS but not with the primary endpoint of OS. We consider this to be potentially influenced by several issues including (i) sample size and its influence on statistical power, (ii) differential drug efficacy between lapatinib and trastuzumab, and (iii) treatment compliance. Design of the original LOGiC trial was probably slightly under-powered (n = 543 in LOGiC compared with 594 in ToGA), as LOGiC was originally based on PFS as the initial primary endpoint, which was later changed to an OS primary endpoint. After exclusion of patients whose disease was HER2-not-amplified, the PEP was limited to 487 participants. LOGiC might have benefited from enrollment of additional participants to gain statistical power for an association with OS. Second, the differential in median OS favored the ToGA trial (ToGA: 13.8 vs. 11.1 months; compared to LOGiC: 12.2 versus 10.5 months). However, it is unknown what role treatment compliance may have played in the clinical outcomes. LOGiC may have had a lower rate of compliance with experimental drug treatment. In contrast to trastuzumab with the requirement for intravenous administration, lapatinib, an oral medication, was self-administered by most participants. This introduces the potential for reduced compliance in the lapatinib treatment arm, especially among patients who may have experienced gastrointestinal side-effects. These issues are compounded by our observations, reported here, that HER2 gene amplification levels may impact clinical outcomes.

Finally, we have not analyzed the potential for other concurrent alterations to effect treatment resistance. For example, coamplification of HER2 and MET is relatively common in esophago-gastric carcinomas (1, 51) and is associated with lack of treatment response to either MET kinase inhibitor (crizotinib) or HER2 inhibitor (trastuzumab) treatment but is associated with therapeutic response to combined treatment in patients with advanced esophago-gastric carcinomas (51). A comprehensive assessment of genetic alterations in cancers from patients in the LOGiC trial would potentially provide important information about co-occurring alterations associated with HER2 amplification and lack of treatment response.

In summary, HER2 status determined by IHC and FISH was highly correlated in UGI cancers. The prevalence of HER2 gene amplification was slightly higher in UGI cancers from men compared to women and was also higher among older patients. HER2 genomic heterogeneity was relatively infrequent, approximately 1.6% overall. The level of HER2 amplification was also correlated with responsiveness to lapatinib treatment in UGI cancer patients.

Disclosure of Potential Conflicts of Interest

M.F. Press reports receiving a commercial research grant from GlaxoSmithKline and is a consultant/advisory board member for Daku, GlaxoSmithKline, Pama Biotechnology, Inc., and Cepheid. M. Bayes is a chief scientific officer and has ownership interest (including patents) in IDDI. I. Villalobos reports receiving a commercial research grant from GlaxoSmithKline and is a consultant/
advisory board member for Dako, GlaxoSmithKline, Puma Technologies, Inc., and Gepheid. Y.-J. Bang is a consultant/advisory board member for GlaxoSmithKline. J.R. Hecht is a consultant/advisory board member for Genentech.

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Acknowledgments

The authors would like to thank the patients who enrolled in the trial as well as the TRIO/Cancer International Research Group Investigators who recruited them at each of the clinical sites and staff at those sites that supported the trial (see Supplementary Data). The authors thank Angela Santiago (USC central laboratory), Roberta Guzman (USC central laboratory), Arshen Gasparian (USC central laboratory), Andreas Marx (University of Hamburg central laboratory), Silvia Schnoeger (University of Hamburg central laboratory), Sascha Eghesassi (University of Hamburg central laboratory), Sina Dietrich (University of Hamburg central laboratory) for technical assistance; as well as Mary-Ann Lindsay (TRIO), Valerie Bee (TRIO), and Henry Taupin (TRIO), Allison Kenner (GlaxoSmithKline), Tomomi Kaneko (GlaxoSmithKline), Saba Khan-Wasti (GlaxoSmithKline), Sherry Wang (GlaxoSmithKline) and Yingjie Huang (GlaxoSmithKline) Carmen Arenas-Elliott (GlaxoSmithKline, Oncology R&D) for operational support. Lapatinib has become the property of Novartis Pharma AG as of March 1, 2015.

Grant Support

GlaxoSmithKline provided support to Translational Research In Oncology (TRIO) to fund the clinical trial and the central laboratories to screen clinical specimens for the trial. The conduct of the clinical trial was determined by the Steering Committee. The decision to analyze this data was made by the Translational Research Committee. Both committees contained minority GlaxoSmithKline representation.

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Received November 3, 2015; revised September 12, 2016; accepted October 6, 2016. Published OnlineFirst November 3, 2016.

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Molecular Cancer Therapeutics

HER2 Status in Advanced or Metastatic Gastric, Esophageal, or Gastroesophageal Adenocarcinoma for Entry to the TRIO-013/LOGiC Trial of Lapatinib


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doi:10.1158/1535-7163.MCT-15-0887

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