Targeted Therapies for Targeted Populations: Anti-EGFR Treatment for EGFR-Amplified Gastroesophageal Adenocarcinoma

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ABSTRACT
Previous anti-EGFR trials in unselected patients with gastroesophageal adenocarcinoma (GEA) were resoundingly negative. We identified EGFR amplification in 5% (19/363) of patients at the University of Chicago, including 6% (8/140) who were prospectively screened with intention-to-treat using anti-EGFR therapy. Seven patients received ≥1 dose of treatment: three first-line FOLFOX plus ABT-806, one second-line FOLFIRI plus cetuximab, and three third/fourth-line cetuximab alone. Treatment achieved objective response in 58% (4/7) and disease control in 100% (7/7) with a median progression-free survival of 10 months. Pretreatment and posttreatment tumor next-generation sequencing (NGS), serial plasma circulating tumor DNA (ctDNA) NGS, and tumor IHC/FISH for EGFR revealed preexisting and/or acquired genomic events, including EGFR-negative clones, PTEN deletion, KRAS amplification/mutation, NRAS, MYC, and HER2 amplification, and GNAS mutations serving as mechanisms of resistance. Two evaluable patients demonstrated interval increase of CD3+ infiltrate, including one who demonstrated increased NKp46+, and PD-L1 IHC expression from baseline, suggesting an immune therapeutic mechanism of action. EGFR amplification predicted benefit from anti-EGFR therapy, albeit until various resistance mechanisms emerged.

SIGNIFICANCE: This paper highlights the role of EGFR inhibitors in EGFR-amplified GEA—despite negative results in prior unselected phase III trials. Using serial ctDNA and tissue NGS, we identified mechanisms of primary and acquired resistance in all patients, as well as potential contribution of antibody-dependent cell-mediated cytotoxicity to their clinical benefit. Cancer Discov; 8(6); 696–713. ©2018 AACR.

See related commentary by Strickler, p. 679.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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doi: 10.1158/2159-8290.CD-17-1260
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INTRODUCTION

Gastric and esophagogastric junction (EGJ) adenocarcinoma, together gastroesophageal adenocarcinoma (GEA), has the third highest incidence and second highest cancer-related mortality, and it remains a significant global health problem (1). When routine screening is not conducted, most patients present with de novo metastatic disease or locally advanced disease with high risk of recurrence. Approximately 55% to 60% of patients recur within 5 years after curative-intent resection despite perioperative therapy (2). The median overall survival (OS) of stage IV GEA is 11 to 12 months with optimal palliative chemotherapy and increases to 16 months for patients with HER2-amplified tumors with the addition of trastuzumab to first-line chemotherapy (3). To date, ramucirumab, an anti-VEGFR2 monoclonal antibody, is the only other approved second-line biologic therapy for GEA as monotherapy or in combination with paclitaxel, with modest clinical benefit (4, 5).

Numerous other targeted therapies have been evaluated in metastatic GEA in various lines and settings, but all of these have been uniformly negative. Recent examples include EGFR, MET, mTOR, and hedgehog pathway inhibitors—generally in genomically unselected patients (6–13).

EGFR is a well-recognized mediator of oncogenic phenotype. EGFR inhibitors including monoclonal antibodies (cetuximab, panitumumab, and necitumumab) and tyrosine kinase inhibitors (erlotinib, gefitinib, afatinib, lapatinib, and osimertinib) have been approved for various cancers, including lung, head and neck, and colon. Early-phase clinical trials had suggested potential benefit in unselected patients with GEA (14–26). These results supported further evaluation in larger phase III trials—EXPAND (cetuximab plus chemotherapy, first line), REAL-3 (panitumumab plus chemotherapy, first line), and COG (gefitinib monotherapy, second–fourth lines; refs. 6–8). Disappointingly, each of these phase III trials was negative, and panitumumab actually resulted in worse survival compared with the control; evaluation of EGFR inhibition was abruptly abandoned for GEA.

If HER2-targeted therapeutic development was devised in the same biologically unselected manner, it likely would have suffered the same fate as anti-EGFR therapy—to the detriment of that subset of patients with HER2-amplified tumors who we now understand to derive significant benefit from this targeted approach. In the registration TOGA trial, nearly 4,000 patients were screened in order to identify the necessary number of HER2-amplified patients (n = 584) to adequately power the study. Despite this patient selection hurdle representing only ~15% of GEA, screening for HER2 amplification by FISH enriched for those most likely to benefit based on a strong preclinical rationale (3). Analogously, EGFR amplification reportedly occurs in ~4% of GEA in The Cancer Genome Atlas (TCGA; CbioPortal; refs. 27, 28), yet prospective EGFR amplification screening in stage IV patients and targeting
therapy in this biologically relevant population is currently lacking.

TCGA as well as other sequencing efforts identified a high degree of chromosomal instability in GEA (27–37). This instability generates additional oncogenic drivers, including gene amplifications of well-known receptor tyrosine kinases, including HER2, MET, FGFR2, and EGFR. Preclinical and clinical evidence suggests benefit of EGFR inhibitors for EGFR genomically driven tumors. In lung cancer, patients with EGFR-mutated tumors derive a greater response and survival than EGFR wild-type patients (38, 39). More relevantly, analyses were reported from two large phase III trials for squamous cell lung cancer evaluating the subset of patients with EGFR amplification or increased gene copy number. In this “EGFR-positive” subgroup of patients, the addition of cetuximab to chemotherapy in the SWOG S0819 trial increased median OS from 6.4 to 11.8 months (P = 0.007; ref. 40), and in the SQUIRE trial (41), this same molecular subgroup trended toward a significant benefit with necitumumab [progression-free survival (PFS)/OS: HR, 0.71/0.70; refs. 41, 42). Similarly, Phase II evaluation of second-line or greater icotinib in patients with advanced squamous esophageal cancer with either high expression by IHC or amplification by FISH demonstrated a 16.7% objective response rate and 46.3% disease control rate (43). Most relevant, however, are studies focusing on EGFR gene copy and benefit from anti-EGFR therapy in GEA samples and patients. Preclinical xenograft models of GEA demonstrated that all responders possessed ≥4 EGFR copies and suggested an even smaller population of those with “high” gene copy number having the highest probability of benefit (44). A post hoc subset analysis of a phase II trial of FOLFOX with cetuximab in GEA confirmed an association between EGFR amplification and OS (P = 0.011; ref. 45). In the TRANS-COG correlative study of the prospective phase III COG trial of gefitinib monotherapy, 16.3% of patients possessed “EGFR-positive” tumors, and the smaller subset of these patients with true gene amplification (6.1%, 18/294) derived a statistically significant survival benefit with the addition of gefitinib in a post hoc analysis (HR, 0.19; P = 0.007; ref. 46). The EXPAND trial demonstrated survival benefit in the small subset with extremely high EGFR H-score expression (6, 47), which was possibly attributable to an underlying subset of EGFR-amplified tumors, but this has yet to be confirmed.

EGFR monoclonal antibodies reportedly act by preventing activating ligands from binding the extracellular domain and/or receptor internalization/degradation of the receptor, but induction of antibody-dependent cell-mediated cytotoxicity (ADCC) via the Fc portion of the antibody may serve as an additional mechanism of action (48, 49). This has been reported with multiple IgG1 monoclonal antibodies, including rituximab, trastuzumab, and cetuximab, and is believed to be mediated by natural killer (NK) cells with resultant dendritic cell and CD8+ T-cell priming (48–51).

Due to these preclinical and clinical subset analyses suggesting potential benefit of EGFR inhibitors for patients with EGFR-amplified GEA, we sought to first describe the incidence of EGFR amplification in a large cohort of patients with GEA across stages and to evaluate for a direct correlation of gene copy number with protein expression levels. We then prospectively screened 140 clinic patients with stage IV GEA (in any line of therapy) over 27 months at our center for EGFR amplification and, when present and otherwise eligible, treated with EGFR monoclonal antibody therapy under institutional review board (IRB)-approved protocols, when appropriate. We report their clinical responses and disease control to EGFR blockade as well as characterization of pretreatment and posttreatment tumor biopsies and serial circulating tumor DNA (ctDNA) next-generation sequencing (NGS) in an attempt to explain clinical outcomes and mechanisms of resistance, and to evaluate the contribution of NK cell–dependent ADCC to antitumoral effect.

RESULTS

EGFR Gene Amplification and Protein Overexpression in GEA Cell Lines

EGFR/CEP7 FISH ratio and EGFR-selected reaction monitoring (SRM) mass spectrometry expression were assessed, as previously described (52), in 24 GEA cancer lines, lymphoblast and breast cancer–negative controls, as well as two positive control head and neck cancer cell lines (HN5 and SQ20B) both known to harbor EGFR amplification (Fig. 1; Supplementary Table S1). EGFR was amplified (FISH ratio EGFR/CEP7 ≥2) in only the two head and neck cell lines (Fig. 1A). EGFR-SRM ranged from <100 to 41,383 amol/μg (median 575 amol/μg; Fig. 1C). EGFR/CEP7 ratio ≥2 and SRM values ≥4,000 amol/μg were strongly correlated (Fisher exact test P = 0.002) in the cell lines. No EGFR expression ≥4,000 amol/μg was observed in cell lines in the absence of EGFR amplification by FISH (Supplementary Table S1).

EGFR Amplification and Overexpression in GEA FFPE Tissues

Five hundred two samples from 363 patients in the University of Chicago GEA tumor bank underwent NGS by Foundation
Anti-EGFR Therapy for EGFR-Amplified Gastroesophageal Cancer

A

B

C

D

E

F

G

[Image of figures and graphs]

Anti-EGFR Therapy for EGFR-Amplified Gastroesophageal Cancer

RESEARCH ARTICLE

June 2018

Cancer Discovery

| 699

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Table 1A. Demographics at diagnosis by EGFR amplification status across the entire U of C cohort

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<th>Characteristic</th>
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<th>EGFR nonamplified</th>
<th>P</th>
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<td>344 (95)</td>
<td>&lt;2.2 × 10^{-16}</td>
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<td>63 (16–91)</td>
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<td>Disease site, n (%)</td>
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<td>12 (63)</td>
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<td>Gastric</td>
<td>7 (37)</td>
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<tr>
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<td>34 (10)</td>
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<td>African American</td>
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<tr>
<td>Asian</td>
<td>1 (5)</td>
<td>27 (8)</td>
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<tr>
<td>Hispanic</td>
<td>1 (5)</td>
<td>9 (3)</td>
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</tbody>
</table>

Abbreviation: U of C, University of Chicago.

Table 1B. Demographics by EGFR amplification status across all stage IV or recurrent esophagogastric cases in retrospective U of C cohort, excluding those prospectively screened between September 2014 and December 2016

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>EGFR amplified</th>
<th>EGFR nonamplified</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>10 (7)</td>
<td>134 (93)</td>
<td>&lt;2.2 × 10^{-16}</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>64.5 (37–77)</td>
<td>62 (22–83)</td>
<td>0.83</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>9 (90)</td>
<td>93 (69)</td>
<td>0.28</td>
</tr>
<tr>
<td>Disease site, n (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Esophagus/GEJ</td>
<td>6 (60)</td>
<td>63 (47)</td>
<td>0.52</td>
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<tr>
<td>Gastric</td>
<td>4 (40)</td>
<td>71 (53)</td>
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<tr>
<td>Tumor grade, n (%)</td>
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<td></td>
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<tr>
<td>Well differentiated</td>
<td>0 (0)</td>
<td>4 (3)</td>
<td>0.76</td>
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<tr>
<td>Moderately differentiated</td>
<td>1 (10)</td>
<td>32 (24)</td>
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<td>Poorly differentiated</td>
<td>8 (80)</td>
<td>97 (72)</td>
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<td>1 (10)</td>
<td>1 (1)</td>
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</tr>
<tr>
<td>Stage at diagnosis, n (%)</td>
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<tr>
<td>I</td>
<td>0 (0)</td>
<td>9 (7)</td>
<td>0.94</td>
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<tr>
<td>II</td>
<td>0 (0)</td>
<td>12 (9)</td>
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<td>III</td>
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<td>IV</td>
<td>8 (80)</td>
<td>83 (62)</td>
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<td>0 (0)</td>
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<tr>
<td>HER2, n (%)</td>
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<tr>
<td>Positive</td>
<td>3 (30)</td>
<td>30 (22)</td>
<td>0.74</td>
</tr>
<tr>
<td>Negative</td>
<td>6 (60)</td>
<td>83 (62)</td>
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<td>Equivocal</td>
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<tr>
<td>Race, n (%)</td>
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<tr>
<td>Caucasian</td>
<td>7 (70)</td>
<td>104 (78)</td>
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<td>African American</td>
<td>2 (20)</td>
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<td>1 (10)</td>
<td>9 (7)</td>
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<tr>
<td>Hispanic</td>
<td>0 (0)</td>
<td>0 (0)</td>
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</tr>
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</table>

One and/or SRM mass spectrometry by Nantomics and were included in the overall cohort (Table 1A; Supplementary Table S2A). Among these cases, 18, 183, and 292 patients underwent EGFR FISH (Fig. 1B), EGFR-SRM (Fig. 1D), and NGS, respectively. One hundred twelve patients had both SRM and NGS, and 11 patients underwent testing by all three modalities. There was a statistically significant linear correlation between EGFR copy-number and EGFR expression by SRM (Pearson correlation = 0.87, P < 2.2 × 10^{-16}), with a trend to significance when evaluating binary “presence” or “absence” of amplification versus expression (P = 0.08). EGFR amplification was identified in 19 of 363 (5%) overall patients across all disease stages in both the retrospective and prospective cohorts (Table 1A), 10 of 144 (7%) retrospectively evaluated stage IV patients only (Table 1B), and 8 of 140 (6%) prospectively screened stage IV patients only (Table 1C). Only one EGFR-amplified case was identified in the absence of EGFR expression ≥1,200 amol/μg in formalin-fixed, paraffin-embedded (FFPE) samples (Supplementary Tables S2A–S2C).

All cases with EGFR expression ≥1,200 amol/μg were EGFR amplified.

Incidence of EGFR Amplification and Concurrent Genomic Aberrations in Metastatic GEA

To further define the 6% to 7% EGFR amplification incidence observed in our stage IV cohorts as compared with the incidence noted from the TCGA (4%) comprising earlier stage tumors, we queried the Foundation Medicine database for the incidence of EGFR amplification among all unique patients with GEA (N = 4,645) sequenced with the Foundation One test between 2012 and 2017. These samples were considered, for the most part, to be from...
short variant events in tumor suppressors, including gene copies. Concurrent genomic aberrations occurring in ≥GEA samples (2.6% of all GEA samples) had ≥50 (Table 2A; Fig. 1E). Forty-six percent of copy number was 40 copies with a range of 8 to 375 copies in distal gastric tumors (Table 2A). The median was identified in 5.6% of patients with GEA, with a higher staging information was unavailable.

patients with advanced metastatic GEA; however, detailed staging information was unavailable. **EGFR** amplification was identified in 5.6% of patients with GEA, with a higher rate of 7.1% in proximal EGI tumors as compared with 3.7% in distal gastric tumors (Table 2A). The median **EGFR** gene copy number was 40 copies with a range of 8 to 375 copies (Table 2A; Fig. 1E). Forty-six percent of **EGFR**-amplified GEA samples (2.6% of all GEA samples) had ≥50 **EGFR** gene copies. Concurrent genomic aberrations occurring in >5% of **EGFR**-amplified samples in this dataset were mostly short variant events in tumor suppressors, including **TP53**, **CDKN2A**, **ARID1A**, **SMAD4**, and **CDH1**, and amplifications of various oncogenes, including **MYC**, **ERBB2**, **KRAS**, **CCND1**, and others (Fig. 1F).

**Table 1C. Demographics by **EGFR** amplification status across all stage IV or recurrent esophagogastric cases prospectively screened at U of C for anti-EGFR therapy between September 2014 and December 2016**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><strong>EGFR</strong> amplified</th>
<th><strong>EGFR</strong> nonamplified</th>
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<td>Number of patients (%)</td>
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<td>Median age, y (range)</td>
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<td>61.5 (19–91)</td>
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<td>Male sex, n (%)</td>
<td>6 (75)</td>
<td>95 (72)</td>
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<td>Disease site, n (%)</td>
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<tr>
<td>Esophagus/GEJ</td>
<td>5 (63)</td>
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<td>Gastric</td>
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<td>Tumor grade, n (%)</td>
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<td>0 (0)</td>
<td>4 (3)</td>
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</table>

**Anti-EGFR Therapy for **EGFR**-Amplified Gastroesophageal Cancer**

**EGFR Amplification Is Associated with Lower PD-L1 Expression by IHC**

Given the growing interest and importance of PD-1 and PD-L1 checkpoint inhibition in GEA, we also assessed the incidence of PD-L1 positivity by tumor positivity score (TPS), tumor-infiltrating lymphocytes (TIL), and combined positivity score (CPS) by **EGFR** amplification status (Table 2B; Fig. 1G). Of the 632 patients with GEA in the Foundation Medicine database for whom PD-L1 IHC was performed (N = 632), 26% of samples were CPS score positive (≥1%; see Materials and Methods). **EGFR**-amplified tumors had lower incidence of CPS positivity (17%) compared with nonamplified tumors (27%), which was not statistically significant.

**Clinicopathologic Characteristics of **EGFR** Amplification**

In the overall University of Chicago cohort (N = 363), which comprised 223 retrospectively accrued and 140 prospectively accrued patients, there was no statistically significant difference in gender, race, age, stage, tumor grade, primary tumor location, or HER2 positivity among patients with **EGFR** amplification versus those without amplification (Table 1A). However, among the **EGFR**-amplified patients, 63% had esophageal or junctional tumors whereas 37% had distal gastric tumors, as compared with 53% and 47% in the nonamplified patients, respectively. When evaluating only patients having stage IV and recurrent disease, no statistical differences in gender, race, age, tumor grade, tumor location, or HER2 status were identified in the retrospective (Table 1B) nor the prospective cohorts (Table 1C).

**Anti-EGFR Antibody Therapy for **EGFR**-Amplified Patients**

Eight of the 140 screened patients (6%) during the prospective screening period (September 2014–December 2016) demonstrated baseline tumor tissue **EGFR** amplification (defined as ≥28 copies by NGS) ranging from 54 to 167 **EGFR** gene copies by NGS (Fig. 2A). Evaluation of each patient’s samples for plasma **EGFR** ctDNA (Fig. 2B), **EGFR**/CEP7 FISH (Fig. 2C), along with **EGFR** IHC and PD-L1 IHC (Fig. 2D), was performed. Seven of these 8 patients ultimately underwent at least one dose of **EGFR**-directed therapy (Supplementary Table S3)—three patients received first-line FOLFOX plus ABT-806 (investigational **EGFR** monoclonal antibody inhibitor as part of the PANGEA trial; ref. 53), one received second-line therapy with FOLFIRI plus cetuximab, two received third-line cetuximab monotherapy, and one received fourth-line cetuximab monotherapy (for patient details, see Supplementary Data File S1). The eighth patient, who had concurrent **MET** and HER2 amplification, was not eligible for **EGFR**-directed therapy due to poor clinical condition after failure of first-line FOLFOX therapy and enrollment in hospice.

Objective best response, the primary endpoint, was observed in 57% (4/7) of patients, including complete responses in 43% (3/7), partial responses in 14% (1/7), and disease control in the remaining 43% (3/7; Fig. 3A and B). Complete responses included one patient (patient 3) receiving third-line cetuximab monotherapy who had a durable response of 14 months with resolution of his symptomatic...
cough) pulmonary metastases (Fig. 3C). Median PFS was 10 months (range, 0.5+ to 14; Fig. 3B). Among the seven patients treated, all four radiographic responses were seen in patients with baseline plasma-detected EGFR amplification over the 50th percentile (2.4 copies in plasma), and the degree of plasma copy-number amplification correlated with objective RECIST response, with the mean ctDNA copy number being 2.5 in nonresponders and 33.9 in responders, and mean difference 31.4 copies between responders and nonresponders (P = 0.049, 95% CI, 0.25–62.5; Fig. 3A). Notably, however, patients 5 and 7, both having EGFR amplification observed in only their primary tumors and not metastases, had clinically significant improvements in their dysphagia/dyspepsia only once ABT-806 was added to their chemotherapy (Supplementary Data File S1; Supplementary Fig. S1).

All four patients receiving cetuximab developed a stereotypical acneiform rash (which interestingly continued during treatment benefit, yet resolved by the time of disease progression), whereas the three patients receiving ABT-806 did not; this was consistent with low rash frequency in phase I evaluation of ABT-806 (54). There were no new safety signals with the addition of EGFR monoclonal antibody therapy as monotherapy or in combination with chemotherapy.

**Mechanisms of Resistance to EGFR Blockade**

Underlying baseline and acquired mechanisms of resistance to therapy were evaluated using baseline and serial tumor tissue NGS in parallel with baseline and serial plasma ctDNA NGS in all treated patients, with confirmation by IHC/FISH, when applicable (Table 3; Fig. 4A). Likely mechanisms of resistance existing prior to treatment initiation were identified in seven of eight patients, and included intra- or intertumoral EGFR amplification heterogeneity in five of seven patients (n = 5, patients 1, 2, 5, 7, and 8), as observed by areas with and without EGFR amplification within the primary tumor itself and/or across different tumor sites anatomically. Additional baseline mechanisms of resistance included coamplification of HER2 (n = 3, patients 2, 4, 8), NRAS (n = 1, patient 4), KRAS (n = 1, patient 6), MYC (n = 4, patients 1, 2, 4, and 6), or CCNE1 (n = 2, patients 4 and 6), as well as mutation in KRAS (n = 1, patient 5) or mutation of another stimulatory G-protein alpha subunit, GNAS (n = 1, patient 6; Table 3 and Fig. 4A).

There were two observed groups of patients upon disease progression—those with retained and those without retained tissue EGFR amplification/overexpression (Fig. 4B–E). Serial ctDNA demonstrated a steep decline in EGFR copy number with EGFR-directed therapy in all evaluable patients including those receiving anti-EGFR antibody monotherapy, but eventual recovery and increase was seen in some patients (patients 1 and 3) upon disease progression and development of resistance mechanisms, which correlated with rise in serum CA19-9 (Fig. 4B; Supplementary Fig. S2A and S2C). In a patient with retained EGFR amplification in tissue, acquired PTEN deletion contributed to resistance, along with a de novo PIK3CA mutation identified in ctDNA (patient 1). In contrast, loss of EGFR...
Anti-EGFR Therapy for EGFR-Amplified Gastroesophageal Cancer

Figure 2. Assessment and correlation of baseline tumor tissue EGFR amplification and expression as well as EGFR plasma ctDNA amplification in seven patients treated with anti-EGFR therapy. EGFR status assessment by (A) tumor NGS, (B) ctDNA NGS, (C) EGFR/CEP7 FISH, and (D) EGFR IHC, as well as PD-L1 IHC (Abcam antibody; see Methods). All samples demonstrated concordant tumor/ctDNA (n > 90th percentile, n > 50th percentile in patient 5, where the metastatic biopsy was not EGFR amplified) except patient 7, whose metastatic biopsy was also not EGFR amplified (see Tables 2A and B).

amplification/expression (likely a selection of preexisting but not previously identified EGFR nonamplified clones) was seen in three cases (patients 2, 3, and 5), as well as patient 1 in a separate large region of the primary tumor, whereas patient 7 never harbored systemic EGFR amplification in his metastatic biopsy nor ctDNA. Patient 3 demonstrated persistent EGFR amplification by ctDNA, but his posttreatment biopsies (new lung, residual primary tumor) were not EGFR amplified and...
he developed BRAF, MET, and MYC coamplification in ctDNA (Fig. 4C). He also developed new brain metastases after disease progression that were not biopsied.

**Immune IHC Evaluation Pre- and Post-Anti-EGFR Therapy**

Pretreatment tissue IHC revealed weak tumoral and stromal CD3 staining in all patients with available tissue (Fig. 4F; Supplementary Table S4). Both tumoral and/or stromal CD3 baseline staining persisted in all five available pre/post-therapy biopsy pairs (patients 1–5). Four of the five posttreatment biopsies were performed on treatment while clinically stable, and one at the time of clinical progression.

At baseline, six of seven patients also had NK cells present in the stroma, but only one had baseline tumoral NKp46 cell staining (patient 2). CD3 stromal staining in patient 1 increased from 2+ to 4+ with concomitant increased NKp46 and PD-L1 staining in a biopsy taken during therapeutic response (obtained prior to receiving anti-PD-L1 and anti-CTLA4 combination therapy previously, because a post–immuno-oncologic and pre–anti-EGFR biopsy could not be obtained; Supplementary Table S4). Increased intratumoral CD3 staining was also observed in patient 2. Conversely, patient 2 demonstrated decreased NKp46 stromal cell staining and no tumoral staining in a biopsy obtained after disease progression on anti-EGFR ABT-806 plus FOLFOX therapy.
Of the four biopsies performed on treatment during disease stability, only two had persistent EGFR expression and EGFR amplification. Both of these persistent EGFR-amplified tumors (patients 1 and 4) also expressed PD-L1, whereas patients now lacking EGFR amplification (patient 3) or postprogression and no longer EGFR amplified (patient 5) did not demonstrate PD-L1 expression. Patient 2 also exhibited PD-L1 expression, but only in the moderately differentiated residual HER2+ region of his primary tumor both pre- and posttreatment; yet again not in the poorly differentiated residual HER2+/EGFR- nonamplified component posttreatment, where EGFR-amplified clones were no longer detected. A biopsy of progressing peritoneal carcinomatosis, which was EGFR-nonamplified, harbored stromal CD3+ staining, but absent PD-L1 and NKp46 expression (Supplementary Table S4).

These observations suggest that while deriving clinical benefit from therapy, anti-EGFR ADCC may have elicited a reflexive upregulation of PD-L1 expression in tumor cells, a so-called interferon alpha/gamma T cell–induced immune response (55), yet over time as tumor response occurred (i.e., EGFR-amplified clones eradicated in patients 2, 3, 5 at posttreatment biopsies) the immune response appeared to have dissipated, and therefore PD-L1 expression subsequently downregulated.

**DISCUSSION**

Herein we quantified the incidence of EGFR amplification and consequent significant EGFR overexpression in 24 GEA cell lines and 502 samples from 363 patients with GEA within the University of Chicago Gastrointestinal Tumor Bank, as well as from a large commercial NGS database of 4,645 patients with GEA. We observed no statistically significant differences in clinicopathologic characteristics in patients with EGFR amplification versus those without amplification, other than a higher proportion in proximal EGJ tumors compared with distal gastric tumors, consistent with the known higher incidence of chromosomal instability (CIN) tumors proximally in the TCGA cohort. We then prospectively screened patients for EGFR amplification and treated them with EGFR-targeting agents when possible. As expected, in this relatively large cohort of 140 stage IV patients screened, only 6% of patients were found to be EGFR amplified, slightly higher than the TCGA 4% incidence. Notwithstanding, a demonstrable and robust treatment response and disease control to EGFR antagonists was observed in this select population. Notably, with monotherapy in heavily pretreated patients, two significant responses (one of which was a complete response) were observed, and a third patient had disease control. Moreover, using tumor NGS in parallel with ctDNA NGS allowed identification and understanding of multiple likely baseline and acquired resistance mechanisms, often concurrently within the same patient across and within tumor sites.

None of the 24 available GEA cell lines demonstrated EGFR amplification or extremely high expression by mass spectrometry, as compared with the EGFR-amplified head and neck cell line controls. An effort to establish more EGFR-amplified cell lines and xenografts is needed in order to enhance understanding of this molecular subset of the disease. Notably, none of the patients identified for treatment in this report had peritoneal ascites or pleural effusions—both recognized metastatic sites that are easily accessible and conducive for establishing tumor cell lines (56). Finally, we demonstrated that tissue EGFR amplification correlated well with protein expression by IHC and mass spectrometry in cell lines and tissues analyzed from the same time point and anatomic location, with the caveat of stromal cellular and tumoral molecular heterogeneity affecting this relationship, as previously described with MET and HER2 (57–59).
Figure 4. Molecular and immunologic correlates of EGFR-amplified samples treated with anti-EGFR therapy. A, Comparison of intrapatient and interpatient significant genomic alterations in primary tumor DNA, metastatic tumor DNA, and ctDNA pre/post–anti-EGFR therapy. B, Serial ctDNA EGFR copy number for all treated patients. C, Serial ctDNA for patient 3 demonstrating decline of EGFR copy number when cetuximab was begun followed by rise in EGFR copy number when MET and BRAF amplification arose at the time of radiographic progression. D, Serial ctDNA for patient 2 revealing steep decline in EGFR copy number after FOLFOX and later ABT-806 initiation with cycle 3 followed by rise in KRAS copy number at the time of occult peritoneal disease growth leading to hydronephrosis. E, Serial ctDNA for patient 4 demonstrating sharp decline in EGFR copy number after cetuximab administration with concomitant rise in ERBB2 copy number at the time of clinical progression and progressive liver failure. F, IHC assessment for CD3+, NKp46+, and PD-L1+ cells pre/post–anti-EGFR therapy, demonstrating increased inflammation within primary tumor from patient 1. G, “Genogram” figure of patient 3. A framework detailing predictive factors favoring response to genomic targeted therapy is toward the periphery including intrapatient homogeneity of EGFR amplification therapy; higher EGFR copy number; combination of chemotherapy + EGFR antagonist; fewer concurrent molecular events; monoclonal EGFR antibody use, and increased CD3 infiltration of tumor and stroma. Patient 3 demonstrated homogeneous and high EGFR amplification at baseline, with moderate immune infiltrate, and without concurrent resistance mechanisms. He therefore would be predicted to derive significant benefit, despite being in the third-line setting and with monotherapy; he had a complete response lasting 61 weeks.
Among University of Chicago tissue samples analyzed, EGFR amplification incidence ranged from 5% to 7% across all stages and cohorts, which is consistent with previous reports (28, 46, 60). Regarding incidence of EGFR amplification specifically in metastatic patients, this was similar in the retrospective and prospective stage IV patients (6%), suggesting a reflective prospective cohort. In the overall population, EGFR amplification trended to be more commonly observed in stage IV patients compared with earlier-stage patients (79% vs. 50% \(P = 0.11\), Table 1A). As such, EGFR amplification incidence was slightly higher in our study than the 4% seen in the TCGA GEA cohort, which was based entirely upon early-stage resected specimens. This disparity may also reflect the difference in incidence between esophageal/esophagogastric junction and gastric primary tumor location, as 14% of TCGA esophageal adenocarcinoma cases were EGFR amplified (28). Accordingly, our study was comprised of a majority of proximal tumors (54%), and EGFR amplification incidence was consistent with the TRANS-COG analysis comprised exclusively of esophageal cancers (46). Our findings are also consistent with previous work demonstrating that CIN GEAs, which are more likely to harbor amplifications, tend to have proximal locations (27, 28). The higher incidence in EGJ versus distal gastric adenocarcinoma and the generally higher incidence compared with TCGA was corroborated in the larger Foundation Medicine database of 4,645 GE samples undergoing Foundation One testing (Table 2A). All other pertinent positive/negative clinicopathologic findings (age, grade, HER2 status, gender, site, and race) were similar regardless of EGFR status.

Despite amplification of EGFR being found in only ~5% to 7% of GEAs, with the high global incidence of distal gastric cancer alone, this may represent nearly 50,000 patients diagnosed each year with EGFR-amplified GEA. ALK-positive non-small cell lung cancer represents a similar paradigm, with a 3% to 5% ALK-translocation frequency that has led to the approval of crizotinib, ceritinib, alecitinib, and brigatinib (61–64). In this report, from 140 patients prospectively screened at one treatment center, we identified and treated seven patients with GEA with extreme amplification of EGFR (54–167 gene copies) in tissue biopsies. By chance, we did not encounter any patients with EGFR amplification with tissue gene copies between 8 and 53, but these patients are not uncommon, as demonstrated in the Foundation Medicine GEA cohort (Fig. 1E). As such, clinical benefit, or differential clinical benefit, in this “lower level tissue copy number subset” cannot be determined from our study. However, we did observe that higher plasma ctDNA copy number did correlate with response within our treated cohort.

We demonstrated clinical benefit with anti-EGFR targeted therapy in clinical scenarios that historically have poor response rates to conventional therapies. In particular, three patients were treated with third/fourth-line monotherapy after exhausting all standard therapies. The observed best objective response rate by RECIST was 4 of 7 (57%) patients across multiple lines of therapy, including a complete and durable response lasting 14 months with monotherapy in the third-line setting (patient 3). We noted a median PFS (mPFS) of 10 months overall (nearly double the mPFS of standard first-line chemotherapy). In comparison, the first-line ToGA trial combined trastuzumab with chemotherapy in treatment-naïve HER2-positive patients and achieved a 6.7-month mPFS, and the second-line RAINBOW study of ramucirumab plus paclitaxel achieved a 4.4-month mPFS (3).

In our treated cohort, tumor reduction with clinical benefit was observed in all cases, and the best disease control rate was 100%. Furthermore, as highlighted in the patient therapy summaries (Supplementary Data File 1), subjective quality of life improvements were seen—even in those with the shortest duration of benefit and those with “primary-tumor-only” EGFR amplification. Baseline and serial ctDNA along with DNA from primary, metastatic, and serial tumor biopsies highlighted significant tumor heterogeneity and widespread potential of therapeutic resistance mechanisms in these patients. Resistance mechanisms included regions of tumors at baseline not harboring EGFR amplification and regions without EGFR amplification at the time of clinical progression. Resistance mechanisms also included concomitant amplifications and mutations in genes putatively involved in circumventing EGFR signaling in the setting of anti-EGFR therapy (65–70), which we also observed in the larger Foundation Medicine GEA cohort (Fig. 1F). Patient 2 was observed to harbor both EGFR and ERBB2 amplification with high expression of both, each within two independent regions (50:50 ratio) of his primary tumor, whereas the retroperitoneal lymph node and bone marrow biopsies, as well as ctDNA, at initial diagnosis harbored EGFR amplification and lacked ERBB2 amplification. For this patient, from a standard-of-care perspective, chemotherapy combined with trastuzumab would be indicated (3), but this would not likely have addressed his primarily HER2-negative metastatic burden. In this patient, clinical resistance and progression of peritoneal carcinomatosis after 10 months of anti-EGFR-based therapy corresponded with the rise of a ctDNA KRAS-amplified clone and KRAS amplification confirmed in the progressing peritoneal biopsy; the primary tumor at this time point was no longer EGFR amplified, nor KRAS amplified, but remained HER2 amplified in ~75% of the biopsy (Fig. 4D; Supplementary Fig. S2B). Patient 1, who had both baseline EGFR-amplified and nonamplified regions of tumor, derived benefit from treatment with FOLFIRI and cetuximab, which together effectively controlled all disease including the EGFR-amplified clone for a period of time. However, as demonstrated on repeat biopsy, this therapy eventually selected for an EGFR-amplified clone with concurrent downstream PTEN exon 6 deletion, along with persistence of the previously identified EGFR-nonamplified region (Supplementary Fig. S2A), as well as a de novo PIK3CA mutation in the ctDNA. Cetuximab resistance via loss of PTEN has been previously demonstrated in colorectal cancer (71). Of note, this deletion was not detected by ctDNA, but rather by endoscopic biopsy and tumor NGS, and this highlights a potential challenge of detecting larger segment deletions in ctDNA. Patient 3 initially had homogenously EGFR-amplified disease, but after 14 months developed a combination of EGFR-nonamplified regions along with presumably EGFR-amplified and concurrent BRAF-, MET-, and MYC-amplified clones that circumvented EGFR inhibition (Fig. 4C; Supplementary Fig. S2C). His persistent EGFR amplification by ctDNA despite absence of amplification identified within postprogression...
biopsies (of both the residual primary tumor and new lung metastasis) suggests additional nonbiopsied sites harboring EGFR amplification, potentially within new brain metastases that were not biopsied. Similarly, selective pressure with cetuximab led to expansion of preexisting HER2, NRAS, and MYC-amplified subclones and emergence of de novo GNAS mutation in patient 4, which all likely conferred therapeutic resistance in various sites within the patient (Fig. 4E; Supplementary Fig. S2D). Patient 5 had baseline MET coamplification as well as KRAS mutation in different anatomic locations. In this patient, significant clinical benefit of anti-EGFR therapy was reported despite lack of RECIST response, with improved local esophagogastric symptoms of pain/dysphagia, which was potentially explained by the EGFR amplification identified only in the primary tumor. Notably this patient’s posttreatment residual primary tumor biopsy no longer identified an EGFR-amplified region. Again, as in patient 1 above, this heterogeneity highlights the benefit of concurrent combination chemotherapy in suppressing other preexisting or acquired resistant clones that would otherwise progress at an accelerated rate if treated with anti-EGFR monotherapy. Patient 6 was unable to obtain drug after cycle 1 due to insurance denial, but presumably would have a more limited benefit in the face of preexisting KRAS/MYC/CCNE1 amplifications and GNAS mutation in the ctDNA at baseline. Her best response was short-lived stable disease after one dose of cetuximab, yet somewhat impressively after disease progression on first-line FOLFOX and second-line paclitaxel/rumucitumab. Finally, patient 7 further highlights the intratumoral and intertumoral heterogeneity of EGFR amplification, as only a fraction of his primary tumor exhibited EGFR amplification, but not in the liver metastasis nor ctDNA. The patient did however derive significant benefit, similar to patient 5 above, as demonstrated with improved dysphagia only after anti-EGFR therapy was added after 4 cycles of ineffective standard FOLFOX chemotherapy (see Supplementary File S1). These seven cases highlight the utility of a comprehensive approach to EGFR-directed combination therapy and other targeted and immuno-oncologic agents combined for optimal tumor control.

A limitation of this study in terms of defining benefit from anti-EGFR therapy is the combination of anti-EGFR therapy with chemotherapy in 4 of the 7 patients. The individual contribution of cetuximab/ABT-806 in combination with chemotherapy is therefore difficult to discern in this small cohort. However, the median PFS with FOLFIRI in second line is only 4 to 5 months (72). Also, clinical benefit in patient 7 was not experienced with four cycles of FOLFOX, and only after the addition of anti-EGFR ABT806 antibody at cycle 5 (when biological grouping was determined on PANGEA study; see Supplementary Fig. S1) was dysphagia dramatically improved, which avoided further intervention including stent and/or palliative radiation. Data regarding the prognostic significance and natural progression of EGFR amplification remain unknown, but EGFR amplification and EGFR overexpression have been associated with shortened survival in some reports (73, 74). Strikingly, however, all three patients who received late-line cetuximab monotherapy began their therapy approximately 2 years after initial stage IV diagnosis—double the median OS in this cancer (Supplementary Table S3). This may suggest that EGFR amplification portends a relatively favorable prognosis, but further larger studies will need to sort this out. It should be noted that patient 3 refused surgery while locally advanced, and therefore the duration of “first-line” therapy in this case is distorted (he was stage IV to the lung by the time of initiating “third-line” monotherapy cetuximab). Despite this, these late-line patients 3, 4, and 6 all demonstrated clinical benefit from cetuximab monotherapy, suggesting that this is indeed an actionable alteration. Notably, each of these three patients had identified EGFR amplification in their original stage IV diagnostic samples as well as their profiling just prior to anti-EGFR therapy in later lines, suggesting stability (and dependence) over time of this aberration with standard therapies. In contrast, and interestingly, most patients treated in early lines in combination with chemotherapy in our cohort had evidence of loss of the aberration in all or at least some of their tissue/plasma samples and/or acquisition of likely concurrent resistance mechanisms (e.g., PTEN deletion) after experiencing disease progression on anti-EGFR therapy. This again confirms the specific targeting of anti-EGFR therapy toward EGFR-amplified clones, with consequent EGFR-amplified clonal eradication and/or pressure to select for concurrent circumventing alterations. Previous phase II and III trials (including COG with gefitinib and EXPAND using cetuximab) demonstrated an OS benefit in the small subset of patients with EGFR-amplified (TRANS-COG) or overexpressed (EXPAND) GEA. This was despite an unimpressive response rate (15, 46), particularly in the TRANS-COG analysis. Of 13 patients in TRANS-COG with EGFR amplification who received gefitinib, none had an objective response. Our results from seven EGFR-amplified patients treated with anti-EGFR monoclonal antibodies suggest a similar benefit, with four patients having durable PFS of over 6 months, but also a high response rate, even in those treated with monotherapy (2/3, 66%), with minimal adverse drug reactions. The difference in response rates between TKI and antibodies is intriguing and although it has a number of possible explanations, it could be explained by ADCC and/or receptor internalization/downregulation, which is not seen with TKIs. Antibody therapy was intentionally chosen for treatment in our study due to potential for ADCC via NK cells seen with cetuximab and other IgG1 monoclonal antibodies, such as ABT-806, as well as less toxicity in combination with chemotherapy relative to TKIs. On the other hand, panitumumab, an IgG2 antibody, may act via myeloid cell lineage ADCC (75, 76), and differences/similarities between these two IgG classes with respect to ADCC are not well delineated, certainly so for EGFR-amplified GEA. Regardless, in head and neck cancer, cetuximab stimulates NK cell recruitment and IFNγ secretion, which mediates dendritic cell maturation and cross-presentation to cytotoxic T lymphocytes against EGFR (77). IFNγ and its associated genes are currently under evaluation as a predictive biomarker of response to PD-1 and PD-L1 antagonists due to their association with a T cell–inflamed tumor environment (78–82). Interestingly, from a large Foundation Medicine cohort of GEA samples undergoing PD-L1 IHC testing, we observed a slightly lower rate of positivity by TPS, TILs, and CPS scoring in EGFR-amplified tumors as compared with nonamplified
tumors (Table 2B; Fig. 1G). A limitation of this analysis is the use of the Ventana SP142 PD-L1 antibody as opposed to the 22C3 pharmDx companion antibody, which was recently approved for pembrolizumab in PD-L1-expressing patients. SP142 has lower sensitivity and therefore possibly underestimates PD-L1 expression (83–85). Regardless, relatively lower frequency of PD-L1 expression by EGFR-amplified tumors compared with nonamplified tumors as we observed in the large Foundation Medicine cohort, if confirmed, may correspond to lower responses to anti-PD-1/anti-PD-L1 checkpoint inhibitor mono-therapy in EGFR-amplified patients. This requires further investigation.

To evaluate the effect of anti-EGFR antibodies on the tumor immune environment, including evidence of ADCC, we evaluated pretreatment and posttreatment tumor biopsies for EGFR, NKp46, CD8, and PD-L1 expression when possible. In this study, results from “during therapy” biopsies imply that treatment with EGFR-directed monoclonal antibodies led to increased tumoral infiltration by CD3⁺ T cells and NKp46⁺ NK cells as well as increased PD-L1 expression, which suggested that consequent ADCC may create, or “trigger,” a reflexive immunosuppressed tumor environment. On-treatment PD-L1 expression appeared more common in cases with persistent EGFR amplification, which also supports this proposed mechanism. Furthermore, patient 1 had previously received, though not responded to, CTLA4 and PD-L1 combination inhibition, and so we cannot detail if his increased post-therapy CD3/PD-L1 staining represents a delayed effect from prior immunotherapy alone, anti-EGFR ADCC alone, sequential immunotherapy and targeted monoclonal antibodies, or spatial heterogeneity. Therefore, although suggestive, these results are limited due to the low sample size and temporospatial biopsy variability. These hypothesis-generating findings merit further prospective investigation to tease out the individual contributions of canonical EGFR ligand–binding inhibition and receptor internalization/ degradation versus ADCC/immune phenomena. Should further studies confirm an upregulation of PD-L1 in anti-EGFR antibody–treated EGFR-amplified tumors, combination with PD-L1 checkpoint blockade would be an appealing combination strategy.

In summary, we report EGFR amplification with overexpression in 5% (19/363) of a large GEA patient cohort. Prospectively, 6% (8/140) of stage IV advanced patients demonstrated EGFR amplification, of which seven patients were successfully treated with at least one dose of anti-EGFR monoclonal antibody therapy. A 57% objective response rate and 100% disease control rate was observed. Within our cohort, elevated plasma-based ctDNA NGS copy-number estimation correlated with objective response by RECIST criteria. This is consistent with a similar-sized prospective study of HER2 amplification in plasma associated with an 80% response rate to targeted therapy in GEA (86, 87). It is likely that response to anti-EGFR therapy will be optimized for depth and duration with the following contributing factors (quite analogous to anti-HER2 for HER2 amplification and likely also to anti-MET for MET amplification and anti-FGFR2 for FGFR2 amplification): (i) homogeneity of EGFR gene amplification within and across all sites of a patient’s tumor burden versus heterogeneous “EGFR-negative” sites, (ii) higher EGFR gene copy number versus lower, (iii) concomitant chemotherapy for synergy on EGFR-amplified clones as well as simultaneous suppression of EGFR-negative clones versus anti-EGFR monotherapy, (iv) lack of baseline genomic resistance mechanisms versus “molecular chaos,” (v) ADCC mechanism of monoclonal antibodies as compared with TKI lacking this mechanism of action, with the patient’s general immune status playing an important role, and (vi) addition of concurrent PD-1/PD-L1 checkpoint blockade to increase immune response. This has been conceptualized in a “genogram,” or “EGFR ampligram,” akin to the recently suggested “immunogram” (55), to serve as a framework to predict clinical benefit from anti-EGFR therapy in EGFR-amplified tumors (Fig. 4G; Supplementary Fig. S3). The degree to which each of these variables contributes to predicted response and response duration will require further investigation. Further assessment of anti-EGFR treatment for EGFR amplification is warranted. Given the relatively low frequency of EGFR amplification, not to mention the issues with intrapatient heterogeneity, evaluation in a traditional phase III study has been elusive and remains difficult, as demonstrated in all phase III EGFR-directed GEA trials to date having only small subsets to evaluate this event, without definitive practice-changing results. Novel trial designs, such as PANGEA, a type II expansion platform design trial in GEA, test a treatment strategy of cytotoxic therapy plus matched targeted therapies across a number of biological subgroups, including EGFR amplification. This design may optimally identify and treat these low incidence aberrations as well as addressing the various mechanisms of resistance at baseline and progression over time (53, 59, 88, 89).

**METHODS**

**GEA Clinical Samples and Cell Lines**

Retrospective and prospective GEA patient samples, with linked clinical and pathological correlates, were obtained from the University of Chicago (Chicago, IL) under IRB-approved tissue banking protocols. This work was conducted in full concordance with the principles of the Declaration of Helsinki. All patients provided written informed consent, where applicable. The human GEA lines and lymphoblast/breast cancer-negative controls were obtained and cultured as previously described (57, 90). The genetic identity of parental cell lines was authenticated by short-tandem repeat profiling (Cell ID System; Promega) at 10 different loci not fewer than 2 months before profiling and experiments. Cell lines tested negative for Mycoplasma contamination with the VenorGen Classic Kit (Minerva Biolabs). These included AGS, CAT-2, CAT-3, CAT-4, CAT11B, CAT12, CAT13, CAT14A, CAT15pl, CP-A, CP-B, CP-C, CP-D, GM14667, HGC-27, Hs746T, KATO III, MKN-1, MKN-45, NCI-N87, OE19, OE33, SNU-1, SNU-16, SNU-5, and ZR-75-30 obtained between 2008 and 2012. The head and neck cancer lines (HNS and SQ20B) were graciously provided by Dr. Ezra Cohen (UCSD) in 2012. CAT lines were established between 2009 and 2016 from malignant ascites or pleural effusion aspirates from patients at the University of Chicago under preapproved guidelines and IRB protocols.

**EGFR FISH**

FISH results for cell lines and retrospective samples included mean EGFR and CEP7 copies/nucleus and EGFR/CEP7 ratio as previously described (90–92) and prospectively screened patients using ClariNet Diagnostics Services Inc. FISH amplification was defined as EGFR/CEP7 ratio ≥2 in all settings.
Sample Preparation and EGFR-SRM Assay
Laser microdissection isolated tumor cells were obtained from FFPE tumor sections as previously described (52, 57, 58, 92, 93). Total protein content for lysates was measured using the Micro-BCA assay (Thermo Fisher Scientific Inc.). The EGFR-SRM assay followed previously described methods and quantified expression in attomols/ microgram (amol/µg; ref. 93).

EGFR Copy Number by Tissue NGS
All NGS results were obtained through routine clinical testing using Foundation One (94); EGFR amplification was defined as EGFR copy 28. Equivocal amplification as noted in the clinical report (copy number 6–7) was considered EGFR nonamplified in this study.

PD-L1 IHC from Foundation Medicine Cohort
GEA samples having PD-L1 testing through Foundation Medicine were identified for analysis (N = 632). PD-L1 testing was performed using the Ventana antibody (SP142) as previously described (95) and was scored three ways: % TPS, % TILs, and combination of two of these for a CPS given recent approval for third-line therapy with pembrolizumab for PD-L1–positive GEA tumors as defined by CPS score ≥1% (83–85).

Quantitative Analysis and Validation of EGFR in Clinical GEA Tissues and Cell lines
EGFR-SRM for 225 retrospective GEA FFPE samples and 28 cell lines was performed by Nantomics and expression was calculated from the ratio of AUC for the endogenous and isotopically labeled standard peptide multiplied by the known amount of isotopically labeled standard peptide spiked into the sample before analysis, as previously described (93).

Identification and Treatment of EGFR-Amplified GEA Patients with Anti-EGFR Therapy
Patients at the University of Chicago with metastatic GEA (any line of therapy) were prospectively screened for EGFR amplification between September 2014 and December 2016 with NGS using the Foundation One test (Foundation Medicine). When remaining tissue was available, EGFR amplification identified by NGS was confirmed by FISH (Clarient Diagnostics Services Inc.). EGFR overexpression was confirmed with IHC (Invitrogen, Clone 31G7, Ventana Ultra View Detection Kit), EGFR-SRM through Nantomics (93). All assays used were Clinical Laboratory Improvement Amendments (CLIA) certified.

Treatment Assignment
Newly diagnosed metastatic, or recurrent after previous curative intent surgery, first-line patients were treated on the PANGEA protocol (NCT02213289; ref. 53) with anti-EGFR antibody ABT-806 24 mg/kg q2 weeks i.v., in combination with FOLFOX per protocol (Supplementary Fig. S1). Otherwise, patients were treated with off-label cetuximab 500 mg q2 weeks i.v. (96), in combination with FOLFOX in the second line, or as monotherapy in the third- and fourth-line settings.

Clinical Outcome Assessment
The primary objective of this study was clinical response as assessed by CT using RECIST 1.1 (97). Measurements were performed independently by clinical interpreting radiologists. Secondary endpoints included PFS and toxicity.

Circulating Cell-Free DNA NGS
Circulating free DNA (cfDNA) sequencing was obtained at baseline prior to anti-EGFR therapy and serially monitored by Guardant 360 in order to correlate cfDNA levels and genomic findings with initial response outcomes and for potential mechanisms of resistance over time (98–100). Absolute plasma copy number was determined utilizing the mode of the normalized number of cfDNA fragments covering each gene to estimate the fragment number corresponding to two copies to derive a baseline diploid value. All values of unique fragments for each gene were then normalized by this baseline value. The baseline derivation was informed by molecule counts data from a large set of normal samples from healthy donors’ plasma. Note that the plasma copy number was related to two variables—the copy number in the tissue and the amount of shedding of tumor DNA into the blood where the tumor DNA—and thus the copy number was expected to be diluted by abundant leukocyte-derived EGFR fragments, the latter having a copy number of 2.0. Centiles of EGFR copy number reported in the clinical G360 results were denoted by a “+” for absolute plasma copy number greater than 2.14 (<50th percentile), “++” for copy number greater than 2.4 but less than 4 (<90th percentile), or “+++” for copy number greater than 4 (290th percentile). In this study, we reported absolute plasma copy number, not these percentiles.

ADCC
The contribution of ADCC was assessed in the prospectively identified anti-EGFR-treated EGFR-amplified cohort by IHC using pretreatment and posttreatment IHC for CD3 (Agilent A0452), NKp46 (R&D Systems, Clone 195314), and PD-L1 (Abcam ab205921) in order to evaluate for treatment-related tumor–stroma modulation.

Statistical Analysis
Comparisons between EGFR-amplified and nonamplified cases were performed using χ² testing or a two-sided Fisher exact test. The relationship between EGFR amplification and expression by SRM or RICIST response was evaluated using the Student t test and by linear regression. PFS was estimated by the Kaplan–Meier method.

Disclosure of Potential Conflicts of Interest
R.B. Lanman has ownership interest (including patents) in Guardant Health, Inc. T. Hembrough has ownership interest (including patents) in NantOomics, LLC. A. Schrock has ownership interest (including patents) in Foundation Medicine, Inc. D.V.T. Catenacci reports receiving commercial research support from Genentech/Roche, has received honoraria from the speakers bureaus of Genentech/Roche, Merck, and Lilly, and is a consultant/advisory board member for Genentech/Roche, Amgen, Lilly, Merck, BMS, and Geistone. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors would like to acknowledge Kyle Holen, Brian Panzl, James Ward, Vincent Blot, and Earl Bain from AbbVie Pharmaceuticals. This work was supported by the NIH (K23 CA178203 and P30 CA014599), University of Chicago Comprehensive Cancer Center (UCCCC) Award in Precision Oncology, Live Like Katie (LLK) Foundation Award, Castle Foundation Award, and the Sal Ferrara II Fund for PANGEA (to D.V.T. Catenacci).

Received November 9, 2017; revised January 11, 2018; accepted February 9, 2018; published first February 15, 2018.

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CANCER DISCOVERY

Published OnlineFirst February 15, 2018; DOI: 10.1158/2159-8290.CD-17-1260


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Steven B. Maron, Lindsay Alpert, Heewon A. Kwak, et al.

Cancer Discov 2018;8:696-713. Published OnlineFirst February 15, 2018.

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