RESEARCH BRIEF

EGFR and MET Amplifications Determine Response to HER2 Inhibition in ERBB2-Amplified Esophagogastric Cancer

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ABSTRACT

The anti-HER2 antibody trastuzumab is standard care for advanced esophagogastric (EG) cancer with ERBB2 (HER2) amplification or overexpression, but intrinsic and acquired resistance are common. We conducted a phase II study of afatinib, an irreversible pan-HER kinase inhibitor, in trastuzumab-resistant EG cancer. We analyzed pretreatment tumor biopsies and, in select cases, performed comprehensive characterization of postmortem metastatic specimens following acquisition of drug resistance. Afatinib response was associated with coamplification of EGFR and ERBB2. Heterogeneous 89Zr-trastuzumab PET uptake was associated with genomic heterogeneity and mixed clinical response to afatinib. Resistance to afatinib was associated with selection for tumors lacking EGFR amplification or with acquisition of MET amplification, which could be detected in plasma cell-free DNA. The combination of afatinib and a MET inhibitor induced complete tumor regression in ERBB2 and MET coamplified patient-derived xenograft models established from a metastatic lesion progressing on afatinib. Collectively, differential intrapatient and interpatient expression of HER2, EGFR, and MET may determine clinical response to HER kinase inhibitors in ERBB2-amplified EG cancer.

SIGNIFICANCE: Analysis of patients with ERBB2-amplified, trastuzumab-resistant EG cancer who were treated with the HER kinase inhibitor afatinib revealed that sensitivity and resistance to therapy were associated with EGFR/ERBB2 coamplification and MET amplification, respectively. HER2-directed PET imaging and cell-free DNA sequencing could help guide strategies to overcome the emergence of resistant clones.

See related commentary by Klempner and Catenacci, p. 166.
INTRODUCTION

Esophagogastric (EG) cancer represents a substantial global health burden, with over a million patients diagnosed worldwide each year (1). The incidence of EG cancer is rising in developing countries, particularly in younger people (2). Up to 30% of EG tumors harbor ERBB2/HER2 amplification or overexpression (3–7). For these patients, trastuzumab (a HER2-directed antibody) improves survival when combined with first-line chemotherapy (3).

Although trastuzumab improves survival in patients with ERBB2-amplified EG cancer, disease progression typically still occurs within a year (3, 8). Therefore, novel treatment approaches are urgently needed for trastuzumab-resistant EG cancer. Unfortunately, the combination of trastuzumab and pertuzumab, an antibody that inhibits HER2 dimer formation, failed to improve survival in this population (9). Furthermore, neither lapatinib (a reversible EGFR/HER2 kinase inhibitor) nor trastuzumab emtansine (T-DM1, an antibody–drug conjugate consisting of trastuzumab linked to the cytotoxic agent DM1) were effective in patients with trastuzumab-refractory EG cancer (10, 11). However, improved outcomes were observed in subsets of patients with high tumor HER2 expression or ERBB2 amplification detectable in plasma cell-free DNA (cfDNA) treated with lapatinib plus chemotherapy (11, 12). Resistance to lapatinib therapy has been associated with reactivation of EGFR and HER3 kinases (13, 14), and the irreversible pan-HER kinase inhibitor afatinib blocks HER2 (14 nmol/L), EGFR (0.5 nmol/L), as well as HER4 (1 nmol/L) kinase reactivation and can induce durable tumor regression in preclinical models of ERBB2-amplified EG cancer (14–17). Dual blockade with trastuzumab and lapatinib has also been shown to induce prolonged inhibition of HER3 and EGFR (14), mirroring the effect of single-agent afatinib, providing a rationale for combining HER2-directed therapies, an approach that is effective in patients with ERBB2-amplified breast cancer (18).

Building on these prior clinical and laboratory observations, we conducted a phase II study to determine the efficacy and safety of afatinib and afatinib/trastuzumab in patients with trastuzumab-refractory ERBB2-amplified EG cancer. Genomic analysis of tumor samples collected before treatment and at disease progression as well as functional imaging with $^{89}$Zr-trastuzumab PET uncovered potential determinants of sensitivity and resistance to therapy. Moreover, in select patients who initially responded to afatinib but later progressed on therapy, we performed research autopsies to allow for analysis of multiple metastatic disease sites in order to explore the molecular basis for heterogeneous responses to afatinib.

RESULTS

Study Design and Summary of Clinical Outcomes

Patients were enrolled between March 2012 and April 2016. Eligible patients had a diagnosis of HER2-positive (IHC 3+, HER2:CEP17 FISH ratio ≥ 2.0) metastatic esophageal, gastroesophageal (GE) junction, or gastric adenocarcinoma with disease progression on at least one trastuzumab-containing regimen. Other inclusion criteria included RECIST 1.1 measurable disease, adequate performance status, and organ function. After the first 10 patients were enrolled, we noted that loss of HER2 was a potential mechanism of trastuzumab resistance (8), and thus the protocol eligibility was amended to mandate confirmation of HER2-positive status by IHC 3+, HER2:CEP17 FISH ratio ≥ 2.0, or next-generation sequencing (NGS) through analysis of a tumor sample collected following progression on trastuzumab and prior to afatinib therapy. In 3 patients treated with afatinib monotherapy (patients 4, 13, and 17), HER2 status following trastuzumab resistance could not be established because the repeat biopsy yielded inadequate tumor content (HER2 assessment details for each patient are provided in Supplementary Table S1). Dose reductions, management of toxicity, and criteria for treatment discontinuation are summarized in Supplementary Methods. Afatinib monotherapy was well tolerated, with diarrhea and skin rash the most common treatment-related adverse events. Grade 2 diarrhea requiring dose reductions was observed in 42% of patients treated with afatinib/trastuzumab despite the prophylactic use of loperamide. A more detailed summary of the adverse effects of afatinib and afatinib/trastuzumab is available in Supplementary Table S2. Detailed clinical information for all patients and tumor samples is given in Supplementary Table S3.

Twenty patients were treated with afatinib monotherapy administered orally at a dose of 40 mg daily. Five of these patients (25%) had tumor reduction according to RECIST 1.1 criteria (Fig. 1A). However, because of the short duration of response in some patients, the RECIST 1.1 objective partial response rate was only 10% (2 of 20 patients). The median progression-free survival was 2 months (95% CI, 1.8–3.51 months), with 3 patients (15%) progression-free at 4 months (95% CI, 3%–33%). Median overall survival for patients treated with afatinib was 7 months (95% CI, 3–11 months).

Given the modest clinical benefit of afatinib monotherapy and preclinical data in patient-derived xenograft (PDX) models suggesting greater efficacy with a HER kinase inhibitor/trastuzumab combination (13–15), a second cohort of 12 patients was treated with afatinib 30 mg daily in combination with trastuzumab 4 mg/kg every 2 weeks. One patient (8%) treated with the afatinib/trastuzumab combination achieved a RECIST 1.1 partial response, and 2 (17%) had disease control for ≥ 4 months (95% CI, 2.6%–41.3%). This was a heavily pretreated population. Across the afatinib and afatinib/trastuzumab cohorts, 75% and 33% of patients, respectively, had progressed on 2 or more trastuzumab-based combination therapies prior to study enrollment (see baseline patient characteristics in Supplementary Table S4 and prior treatment details in Supplementary Table S5).

Tumor Regression on Afatinib Is Associated with Coamplification of ERBB2 and EGFR

To identify molecular determinants of afatinib response, we performed biopsies after trastuzumab therapy but prior to initiation of afatinib or afatinib/trastuzumab in all patients. Only 24 of 32 post-trastuzumab progression biopsies (75%) yielded sufficient tumor material for genomic analysis (Fig. 1A), highlighting the logistic challenges associated with tumor genomic characterization in patients with advanced EG cancer.
Afatinib in \( \text{ERBB2} \)-Amplified Gastric Cancer

**Figure 1.** Individual treatment outcomes of 20 patients treated with afatinib and 12 patients treated with afatinib and trastuzumab. A, Percentage best change from baseline in the target lesion assessed by RECIST 1.1. Relevant clinical features (time on therapy, type of sequenced sample, and collection time point of the sequenced sample) plus key genomic alterations in sequenced samples are shown for each patient. SLD, sum of longest diameter on CT scan. Pt1 to Pt32 are IDs for all the patients in the study. For patients with multiple sequenced samples, the sample included in the oncoprint is designed after a decimal. *, Nonevaluable. **, Sample uninformative due to low tumor content. B, Dual probe \( \text{EGFR} \) and \( \text{ERBB2} \) FISH from patients 9, 30, and 32, all of which were collected prior to protocol treatment, demonstrated diffuse and uniform \( \text{ERBB2} \) and \( \text{EGFR} \) coamplification in virtually all tumor cells. There was no additional tissue for dual probe FISH for patient 31. C, Percent change in \( \text{\textit{\textsuperscript{89}Zr}} \)-trastuzumab PET SUV pre- versus post-therapy for each individual lesion on CT in 1 patient treated with afatinib monotherapy and 7 patients treated with afatinib/trastuzumab. White stars inside the bar plots denote lesions with resolution of uptake to baseline SUV.
For 5 of the remaining 8 patients (63%), pre-trastuzumab tumor biopsies yielded sufficient tumor material for genomic analysis (Fig. 1A). Tumors were analyzed using the MSK-IMPACT assay, a capture-based NGS platform that detects mutations, copy-number alterations, and select rearrangements in up to 468 cancer-associated genes (see Methods; refs. 19, 20). The OncoKB knowledge base was used to distinguish alterations with known functional relevance from variants of unknown significance (21). Notably, the 3 patients with the highest percentage tumor regression on afatinib (patients 30, 31, and 32) had coamplification of ERBB2 and EGFR (mean RECIST 1.1 percent change from baseline of −29% vs. +24%; \( P = 0.025 \), one-sided Wilcoxon rank-sum test; Fig. 1A; Supplementary Table S6). Using dual probe FISH, we confirmed that the EGFR and ERBB2 coamplifications detected by NGS of bulk tumor were the result of concurrent amplification of both genes in the same tumor cells (Fig. 1B; Supplementary Fig. S1). Patients whose tumors harbored KRAS (14%), PIK3CA (10%), or NFI (7%) mutations had rapid disease progression on afatinib monotherapy (Fig. 1A). These data are consistent with prior results demonstrating that mutations in the RAS and PI3K pathways are associated with trastuzumab resistance in ERBB2-amplified EG cancer (8, 12, 22–25).

RAS and PI3K pathways are associated with trastuzumab resistance in patients. (A) Best RECIST responders on afatinib monotherapy (Fig. 1A). These data are consistent with prior results demonstrating that mutations in the RAS and PI3K pathways are associated with trastuzumab resistance in ERBB2-amplified EG cancer (8, 12, 22–25).

Patient 9 was the only nonresponder with EGFR/ERBB2 coamplification. However, afatinib resistance in this case may be explained by co-occurrence of MYC amplification and a truncating mutation in NFI (Fig. 1A). Furthermore, we noticed that EGFR amplification and expression, by dual probe FISH and selected reaction monitoring–mass spectrometry (SRM-MS), respectively, was lower in that patient compared with the responders. The combination of afatinib and trastuzumab did not show greater clinical activity. As was observed in the afatinib monotherapy cohort, patients with comutations in the RAS or PI3 kinase pathways experienced rapid disease progression on afatinib/trastuzumab therapy (Fig. 1A).

Homogenous ⁸⁹Zr-Trastuzumab Uptake on Pretreatment PET May Predict for Therapeutic Sensitivity

We previously reported that ⁸⁹Zr-trastuzumab localizes to HER2-positive EG tumors as measured by PET imaging (26). Furthermore, in contrast to ⁸⁹F-FDG and ⁸⁹F-FLT PET, ⁸⁹Zr-trastuzumab PET could noninvasively differentiate HER2-positive from HER2-negative EG cancers preclinically (15). As downregulation of HER2 expression by afatinib is associated with afatinib sensitivity in preclinical models (15), we hypothesized that functional imaging with ⁸⁹Zr-trastuzumab PET could identify afatinib-sensitive and afatinib-resistant tumor sites prior to evidence of clinical progression. To test this hypothesis, we performed ⁸⁹Zr-trastuzumab PET imaging before treatment and before the first CT assessment (after 3–5 weeks on therapy) in 8 patients, one treated with afatinib monotherapy and seven with afatinib/trastuzumab.

All patients demonstrated ⁸⁹Zr-trastuzumab tumor uptake in at least one disease site prior to therapy initiation. In the pretreatment scans, we observed a wide range in median standardized uptake values (SUV) among patients and among lesions in individual patients (median SUVmax 15.6; range, 6.4–23.8; Fig. 1C; Supplementary Table S7). Four patients (50%) had visible metastases on CT that did not exhibit ⁸⁹Zr-trastuzumab uptake on PET, indicating pretreatment intrapatient HER2 expression heterogeneity among disease sites. All 4 of these patients demonstrated disease progression within 6 weeks. Even when mean ⁸⁹Zr-trastuzumab uptake declined 3 to 5 weeks after therapy, we observed intrapatient lesion-to-lesion variability in this change (Fig. 1C). Notably, the 3 patients with the strongest tumor regression on afatinib and afatinib/trastuzumab therapy had uniformly high ⁸⁹Zr-trastuzumab uptake (SUV >5) in all lesions visualized on CT (see Fig. 1C). These data suggest that homogenous pretreatment ⁸⁹Zr-trastuzumab uptake may be a marker of afatinib sensitivity and that heterogeneous uptake at baseline or variable ⁸⁹Zr-trastuzumab PET response may be indicators of poor response to afatinib in ERBB2-amplified EG cancer.

**Differential Inpatient and Interpatient Expression of the HER2, EGFR, and MET Kinases May Determine Clinical Response to HER Kinase Inhibitors in ERBB2-Amplified EG Cancer**

To further explore the molecular basis of the variable lesion-to-lesion ⁸⁹Zr-trastuzumab uptake observed in individual patients and to identify potential mechanisms of afatinib resistance, we obtained consent to perform whole autopsies on 3 patients (patients 28, 30, and 31) who initially responded to afatinib but later developed disease progression. (B) Pretreatment PET uptake patterns in 5 of the remaining 8 patients, including 3 patients with the strongest tumor regression on afatinib (patients 30, 31, and 32) who showed intrapatient variability in ⁸⁹Zr-trastuzumab uptake (Fig. 1B).
Afatinib in ERBB2-Amplified Gastric Cancer

RESEARCH BRIEF

FEBRUARY 2019}

CANCER DISCOVERY | 203

other liver lesions on both CT and 89Zr-trastuzumab PET imaging.

Following discontinuation of afatinib treatment, the patient clinically deteriorated and died, after which we performed an autopsy to collect tumor tissue from the primary tumor site and all visible metastatic sites (Fig. 2B). MSK-IMPACT sequencing was then performed on all tumor tissues and a matched normal blood specimen to explore the clonal and evolutionary relationships among disease sites. Whereas ERBB2 amplification was clonal and shared among all the sampled disease sites, EGFR amplification was identified in only the pre-afatinib liver biopsy and in only 1 of the 3 tumor sites collected postmortem (liver segment 3).

These results indicate that EGFR amplification defined a distinct subclone that colonized liver segment 2/3 but branched off from other clones before trastuzumab and afatinib therapy (Fig. 2C). Notably, we observed heterogeneity of EGFR copy-number status in two geographically separate regions of the large segment 2/3 liver lesion: one area positive and the other area negative for EGFR amplification. The progressing disease sites at autopsy, although all lacking EGFR amplification and possessing additional private

Figure 2. Intrapatient disease heterogeneity and selection for a non–EGFR-amplified tumor clone as mechanisms of afatinib resistance in patient 30. A, Radiographic tumor assessment using 89Zr-trastuzumab PET and conventional CT scan, with corresponding bar graphs. 89Zr-trastuzumab PET values in SUV and CT measurements in mm. 89Zr-trastuzumab PET images include 3 panels: left (pre-afatinib) is a baseline pretreatment image showing uptake in segment 2/3 liver metastasis (SUV 16.4), GE junction (GEJ; SUV 21.5), and retroesophageal lymph node (RLN; SUV 9.1); middle (on afatinib) corresponds to 3 weeks post-treatment initiation, showing resolution of retroesophageal lymph node (SUV 3.0) with decrease in size but persistent high uptake in segment 2/3 liver lesion (SUV 15.8), similar uptake in GE junction (SUV 23.7), and a new lesion in liver segment 7/8 which appeared on 89Zr-trastuzumab PET (SUV 8.6); right (progression) shows postprogression 89Zr-trastuzumab PET uptake in GE junction (SUV 28.5) and segment 7/8 liver lesion (SUV 9.0), decreased uptake in liver segment 2/3 (SUV 12.3), and ongoing resolution of retroesophageal lymph node. B, Genomic comparison of matched pre- and post-treatment primary and liver segments 2/3 and post-treatment progression liver segment 7/8 obtained at warm autopsy. EGFR amplification was unique to the segment 2/3 liver lesion with ongoing response. The enlarging GE junction tumor and segment 7/8 liver metastasis were not EGFR amplified. In addition to a likely pathogenic mutation in PTEN (P95R, also present in a segment 2/3 liver lesion), the nonresponding metastases acquired an amplification of CCND3. SUV, standardized uptake value on 89Zr-trastuzumab PET; SLD, sum of longest diameter on CT scan. C, Phylogenetic tree showing the inferred patterns of clonal evolution for the samples described in B.
mutations, were clonally related and defined by a likely functional shared mutation in *PTEN* (P95R), and a focal amplification of *CCND3*. In sum, these data suggest that preexistent heterogeneity of *EGFR* amplification may account for the patient’s mixed response to afatinib therapy, with drug resistance arising through selection for a preexistent *EGFR* wild-type, *ERBB2*-amplified clone with additional coalterations of *PTEN* and *CCND3*.

A second patient (patient 31), a 61-year-old with stage IV gastric adenocarcinoma that was metastatic at diagnosis, had a 6-month transient response to first-line fluorouracil/oxaliplatin in combination with trastuzumab. Tumor genomic profiling of a liver metastasis collected post-trastuzumab identified *ERBB2* and *EGFR* coamplification, and the patient subsequently received afatinib monotherapy, achieving a confirmed partial response (best response 43% tumor regression by RECIST 1.1), followed by clinical progression at 12 weeks (Fig. 3). The patient succumbed to the disease within 6 weeks of afatinib discontinuation. Lesions in the primary tumor as well as the liver, pancreas, mesentery, lung, and lymph node.
nodes were collected at autopsy and subjected to tumor molecular profiling. A comparison of the sequencing results from postmortem tumor specimens and the primary tumor biopsy specimens collected prior to afatinib/trastuzumab treatment demonstrated a TP53 R248W mutation in all disease sites. MET amplification was, however, detected only in the post-afatinib progression sites. This MET amplification was further confirmed using FISH (MET/Cen 7 >10; Fig. 3A). SRM-MS analysis of tumor samples collected at autopsy also confirmed uniformly high MET protein expression in all progressing lesions (Supplementary Table S8). Notably, a liver metastasis sampled both during afatinib therapy and at autopsy showed increased MET and HER2 as well as decreased EGFR protein expression in the postprogression versus on-treatment samples (Fig. 3B).

Amplification of MET has been previously proposed as a resistance mechanism to HER2 blockade in HER2-positive gastric cancer (23, 27–29). To determine whether MET amplification was sufficient to confer afatinib resistance in this patient, we studied a PDX model generated from a postprogression sample collected following progression on afatinib. We treated mice bearing this PDX with either afatinib, the MET kinase inhibitor AMG 337, a combination of both agents, or the vehicles only as control. Neither afatinib nor AMG 337 alone had an effect on PDX growth (Fig. 3C). However, the afatinib/AMG 337 combination induced durable tumor regression. By day 21, tumors were no longer palpable ever, the afatinib/AMG 337 combination induced durable tumor control versus on-treatment samples (Fig. 3B).

A third patient (patient 28) had stage IV ERBB2-amplified gastric cancer metastatic to the peritoneal cavity. This patient had a prolonged 24-month response to first-line trastuzumab, fluorouracil, and oxaliplatin. The tumor collected following progression on afatinib/trastuzumab therapy, the patient was treated with afatinib in combination with cabozantinib, a multitargeted kinase inhibitor that inhibits MET among other kinase targets. Despite an initial decline in tumor markers, the patient clinically deteriorated and died. Analysis of multiple disease sites collected at autopsy revealed MET amplification only in the progressing ovarian metastasis and in a new perirectal metastasis not detected by pretreatment imaging. By contrast, the perigastric metastasis and the primary tumor site did not harbor a MET amplification, and both demonstrated ongoing response to afatinib/trastuzumab combination therapy (Fig. 4C). On dual EGFR/ERBB2 FISH, there was heterogeneity within the perirectal metastasis, with all cells demonstrating ERBB2 amplification but a mixture of non-EGFR-amplified and low-level EGFR-amplified cells (Fig. 4D). This heterogeneity was reflected in the observed EGFR amplification as detected by cfDNA (Fig. 4B).

DISCUSSION

We report the results of a phase II study of afatinib monotherapy and combined afatinib and trastuzumab combination therapy in patients with trastuzumab-resistant EG cancer. Integral to this study were analyses of pretreatment tumor biopsies with the goal of identifying determinants of afatinib sensitivity and, in responding patients, evolutionarily analyses of samples collected following disease progression at the time of warm autopsy, to explore the effect of tumor heterogeneity on drug response. We observed that coamplification of EGFR was associated with clinical benefit with afatinib in ERBB2-amplified EG cancer and believe that this hypothesis should be further investigated in a larger cohort of EG patients. Because prior phase II results showed no meaningful response to afatinib in patients with EGFR-amplified EG tumors lacking ERBB2 amplification, our data suggest that patients with coamplification of both ERBB2 and EGFR may be most likely to respond to pan-HER kinase inhibitors such as afatinib (16). Coamplification of ERBB2 and EGFR was not, however, required for afatinib response, as the combination of afatinib with trastuzumab showed modest clinical activity in ERBB2-amplified tumors lacking EGFR amplification. Furthermore, EGFR-selective inhibitors such as cetuximab and gefitinib have demonstrated activity in EGFR-amplified tumors lacking ERBB2 amplification, suggesting that these agents may have a different efficacy profile than afatinib (30–33).

In this study, we performed warm autopsies with the goal of sampling multiple tumor sites to explore the relationship between intrapatient tumor heterogeneity and treatment response. In each case, the paired analysis of drug-sensitive and drug-resistant tumor sites identified a likely resistance mechanism. More specifically, we observed that afatinib resistance was associated with selection for a tumor clone that either lacked a sensitizing amplification (EGFR amplification) or had gained a resistance amplification (MET amplification). The results support the hypothesis that the limited efficacy of targeted kinase inhibitors in EG cancer is the result of rapid selection for or against driver amplifications that are heterogeneous present within an
Figure 4. Noninvasive detection of acquired MET amplification as a mechanism of afatinib/trastuzumab resistance using cfDNA. A, Radiographic tumor assessment using $^{89}$Zr-trastuzumab PET and conventional CT scan, with corresponding bar graphs in patient 28. $^{89}$Zr-trastuzumab PET values in SUV and CT measurements in mm. Left, a baseline pretreatment $^{89}$Zr-trastuzumab PET image showing uptake in a left ovary metastasis (SUV 5.1) not visible in this projection, perigastric peritoneal metastasis (SUV 7.2, lower arrow), and gastric mass (SUV 8.2, upper arrow). Right, $^{89}$Zr-trastuzumab PET 5 weeks post-treatment initiation, showing resolution of uptake in the perigastric peritoneal metastasis (SUV 1.8) and gastric mass (SUV 3.9) and persistently high uptake in the left ovarian metastasis (SUV 5.2) not seen on maximum intensity projection (MIP) images. B, cfDNA analysis at the time of disease progression on afatinib/trastuzumab, demonstrating ERBB2 and MET amplification (results for additional time points are provided in Supplementary Table S9). C, Genomic comparison of matched pretreatment biopsy with post-treatment tumor tissue collected from the left ovarian metastasis and subsequently at rapid autopsy, demonstrating acquired MET amplification unique to the progressing lesions. The perigastric metastasis (met) and the primary tumor, both of which demonstrated ongoing response to afatinib/trastuzumab, did not harbor MET amplification. D, Dual probe EGFR and ERBB2 FISH from a perirectal tumor biopsy collected after treatment demonstrated ERBB2 amplification and low-level gain of EGFR in a subset of tumor cells.

individual patient, a hypothesis consistent with a recently published study of matched primary and metastatic biopsies that noted that amplifications of ERBB2, EGFR, and MET were frequently discordant between primary and metastatic tumor sites (34).

Notably, we were able to generate a PDX model from a drug-resistant tumor site in one of the patients in whom treatment resistance was associated with selection for a MET-complified tumor. Analysis of this PDX model in the laboratory indicated that whereas afatinib and the MET inhibitor AMG 337 had no activity when used alone, cotreatment with both induced complete tumor regression, thus confirming that MET amplification was sufficient to induce afatinib resistance in this patient. Also notably, in one of the 2 patients, we were able to noninvasively detect the MET amplification by analyzing tumor-derived DNA in plasma (cfDNA),
suggesting that cfDNA analysis could noninvasively identify patients for whom EGFR and MET kinase inhibitor combinations would be most appropriate.

In the current study, we also show that HER2-directed functional PET imaging using 89Zr-trastuzumab can noninvasively identify tumor heterogeneity, which may be predictive of subsequent drug response. Although the number of patients imaged was small, we did observe resolution of 89Zr-trastuzumab uptake on PET imaging at some disease sites at early time points with tumor regression at these same sites on subsequent CT scans. These results suggest that HER2-directed PET imaging may represent a potential noninvasive tool to predict therapy response within weeks of therapy initiation. Early increases in 89Zr-trastuzumab uptake may also be useful in identifying treatment-resistant disease sites prior to visible disease progression on traditional CT or MRI imaging. Hence, the potential uses of 89Zr-trastuzumab PET could extend beyond detection of HER2-positive lesions to include facilitation of individualized patient care and management.

In summary, we find that concurrent amplification of EGFR and ERBB2 is associated with response to the HER kinase inhibitor afatinib in patients with trastuzumab-refractory EG cancer. Heterogeneous uptake of 89Zr-trastuzumab measured noninvasively by PET was associated with disease progression. Analyses of multiple disease sites sampled at the time of disease progression indicated several potential mediators of resistance, including loss of EGFR amplification and gain of MET amplification.

METHODS

Study Design and Treatment

This was a single-institution investigator-initiated phase II study of oral afatinib 40 mg daily (cohort 1) or afatinib 30 mg daily with intravenous trastuzumab 4 mg/kg every 2 weeks (cohort 2). The study protocol and all amendments were approved by the Memorial Sloan Kettering institutional review board, and the protocol was conducted in accordance with the Declaration of Helsinki, International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS), the Belmont Report, and the US Common Rule. All patients provided written informed consent. A CONSORT diagram is provided as Supplementary Fig. S2.

Study Assessments

The primary endpoint of the study was overall clinical benefit defined as best overall response rate (ORR; stable disease, complete or partial response) at 4 months. Adverse events were assessed according to National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0.

Biomarkers

To identify predictive biomarkers of response and molecular mechanisms of resistance, NGS, FISH, IHC, and PDX studies were prespecified in the initial version of the protocol, which was later amended to also include 89Zr-trastuzumab PET imaging. Patients were also enrolled to a separate institutional review board–approved tissue procurement protocol, which provided consent for the mass spectrometry, plasma cfDNA, and autopsy studies. cfDNA collected during treatment of patient 28 was analyzed using the Guardant360 assay (35). The MSK-IMPACT assay was performed initially using a 341-gene and later 410-gene and 468-gene panels (Supplementary Table S10), as previously described (8). Multicolor FISH was performed using a probe mix containing equal parts of custom Kreatech MET (7q31) red, EGFR (7p11) green, and ERBB2 (17q12) blue FISH probes (Leica Biosystems). HER2, EGFR, and MET protein levels were quantitated by targeted mass spectrometry (SRM-MS) using the NanOomics processing pipeline, as previously described (36–38). High protein expression was set by SRM level for MET ≥ 1,500 amol/μg, EGFR ≥ 1,200 amol/μg, and HER2 ≥ 750 amol/μg (36–38).

Statistical Analysis

Each treatment group was evaluated separately for the primary endpoint of ORR. Cohort 1 using a Simon two-stage study design (39) with 80% power to reject the null hypothesis of ORR 5% (insufficient drug activity), assuming that the true ORR was 20%, with one-sided α = 0.05. In the first stage, 13 patients were treated with afatinib, and based on 2 responses, accrual was expanded in stage 2 to 19 additional patients. After 20 patients were treated with afatinib, the trial was amended based on synergy observed in preclinical models to combined afatinib and trastuzumab (cohort 2). Cohort 2 was evaluated using the exact binomial single-stage design with unacceptable response rate of 7% against an acceptable response rate of 25% (at least 5 of 26 responding). Accrual was stopped after only 1 response was observed out of 12 patients treated in cohort 2, and because afatinib with trastuzumab was poorly tolerated.

CT and 89Zr-Trastuzumab PET Assessments

CT or MRI scans were performed at baseline and every 8 weeks thereafter with response assessments quantified using RECIST 1.1 criteria. Best overall responses were derived from tumor measurements provided by the study-site radiologist. In patients who were willing to undergo additional imaging, 89Zr-trastuzumab PET imaging was performed before treatment and before the first CT assessment (after 3–5 weeks on therapy) as an exploratory objective to assess early changes on protocol therapy. We have previously reported on the pharmacokinetics and serial biodistribution of 89Zr-trastuzumab in patients with HER2-positive EG cancer (NCT02023996; ref. 26). Trastuzumab conjugated with desferrioxamine and radiolabeled with 89Zr was administered intravenously as previously described (26). The 89Zr-trastuzumab PET images were reviewed together with available conventional CT/MRI for concordance of tumor targeting. Heterogeneity of tumors was assessed on a per-lesion basis by aligning 89Zr-trastuzumab PET and CT/MRI images on a Hermes Medical Imaging workstation. Intrapatient heterogeneity was defined as 89Zr-trastuzumab PET-positive imaging in some but not all lesions identified on CT/MRI (see Supplementary Table S7).

Data Availability

All genomic and clinical data from this study are publicly available through the cBioPortal for Cancer Genomics (http://cBioPortal.org; study?id=egc_msk_afatinib_2018; refs. 40, 41).

Disclosure of Potential Conflicts of Interest

P. Castel has ownership interest (including stock, patents, etc.) in Venthera and is a consultant/advisory board member for Venthera and Quartz Therapeutics. G.Y. Ku reports receiving commercial research support from Pieris and is a consultant/advisory board member for the same. M.E. Lacouture reports receiving a commercial research grant from RJR Fund, reports receiving other commercial research support from Veloce, Berg, US Biotest, and BMS, is a consultant/advisory board member for Boehringer Ingelheim, Roche/Genentech, BMS, AstraZeneca, Amgen, Symphogen, EMD Serono, Janssen, and Novartis, and has received other remuneration from Harborside Press and Wiley. T. Hembrough is President, Proteomics and Quartz Therapeutics. G.Y. Ku reports receiving commercial support from Pieris and is a consultant/advisory board member for Boehringer Ingelheim, Roche/Genentech, BMS, AstraZeneca, Amgen, Symphogen, EMD Serono, Janssen, and Novartis, and has received other remuneration from Harborside Press and Wiley. T. Hembrough is President, Proteomics and Quartz Therapeutics. G.Y. Ku reports receiving commercial support from Pieris and is a consultant/advisory board member for Boehringer Ingelheim, Roche/Genentech, BMS, AstraZeneca, Amgen, Symphogen, EMD Serono, Janssen, and Novartis, and has received other remuneration from Harborside Press and Wiley.
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