Published in final edited form as:

Clin Cancer Res. 2021 March 15; 27(6): 1612–1622. doi:10.1158/1078-0432.CCR-20-1831.

Neratinib-plus-cetuximab in Quadruple WT (KRAS, NRAS, BRAF, PIK3CA) Metastatic Colorectal Cancer Resistant to Cetuximab or Panitumumab: NSABP FC-7, A Phase Ib Study

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Abstract

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Potential Conflicts of Interest:

Dr. George reports grants pending to his institution for Clinical Research Conduct, as follows: Bristol Myers Squibb, Incyte, Merck, Bayer, AstraZeneca/MedImmune, NewLink Genetics, Ipsen, Seattle Genetics, Lilly, and Tesaro/GSK.

Dr. Sama reports employment and stock options with Bristol Myers Squibb

Ms. Piette reports commercial agreement to perform the statistical analyses, to participate in the writing of the statistical section of the manuscript, and the review.

Dr. Pogue-Geile reports grants from the NCI and the Pennsylvania Dept of Health (PA DoH), during the conduct of the study*, and from Puma Biotechnology, Inc. (Puma).

Mr. Lipchik reports grants to his institution from the PA DoH,* and Puma.

Ms. Finnigan reports grants to her institution from the PA DoH,* and Puma.

Dr. Beumer reports expert testimony from Pfizer, and grants from AbbVie, Spectrum, and NCI P30 CA47904 and R50 CA211241.

Dr. Wolmark reports an institutional business contract with Puma.

Dr. Lucas reports consulting fee paid to spouse from Bayer/Loxo, and stock/options with Amgen.

Dr. Allegra reports fees and payments as an employee of NSABP Foundation.

Dr. Srinivasan reports grants to his institution from the PA DoH, and Puma.

* The Pennsylvania DoH specifically disclaims responsibility for any analysis, interpretations, or conclusions

All other authors have no COIs to declare.

Clinical Trials registration: [NCT01960023](https://clinicaltrials.gov/ct2/show/study/NCT01960023)

Purpose: In metastatic colorectal cancer (mCRC), *HER2* (*ERBB2*) gene amplification is implicated in anti-EGFR therapy resistance. We sought to determine the recommended phase II dose (RP2D) and efficacy of neratinib, a pan-*ERBB* kinase inhibitor, combined with cetuximab, in patients with progressive disease (PD) on anti-EGFR treatment.

Experimental Design: Twenty-one patients with quadruple-wild-type, refractory mCRC enrolled in this 3+3 phase-Ib study. Standard dosage cetuximab was administered with neratinib at 120mg, 160mg, 200mg, and 240mg/day orally in 28-day cycles. Samples were collected for molecular and pharmacokinetic studies.

Results: Sixteen patients were evaluable for dose-limiting toxicity (DLT). 240mg was determined to be the RP2D wherein a single DLT occurred (1/7 patients). Treatment-related DLTs were not seen at lower doses. Best response was stable disease (SD) in 7/16 (44%). *HER2* amplification (CISH) was detected in 2/21 (9.5%) treatment-naïve tumors and 4/16 (25%) biopsies upon trial enrollment (post-anti-EGFR treatment and progression). Compared to matched enrollment biopsies, 6/8 (75%) blood samples showed concordance for *HER2* CNV in cfDNA. Five SD patients had *HER2* amplification in either treatment-naïve or enrollment biopsies. Examination of gene-expression, total protein, and protein phosphorylation levels showed relative upregulation of 2 members of the HER-family receptors or ligands upon enrollment versus matched treatment-naïve samples.

Conclusions: The RP2D of neratinib in this combination was 240mg/day, which was well tolerated with low incidence of G3 AEs. There were no objective responses; SD was seen at all neratinib doses. *HER2* amplification, detectable in both tissue and blood, was more frequent post-anti-EGFR therapy.

Keywords

metastatic colorectal cancer; neratinib

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in Western countries(1). It is estimated that there are ~140,000 new CRC cases in the US each year and 50,630 deaths annually. For decades, the mainstay of treatment for both early and advanced disease has been chemotherapy with small incremental gains achieved with targeted therapy including monoclonal antibodies such as anti-VEGF and anti-EGFR pathway inhibitors, and tyrosine kinase (TK) inhibitors such as regorafenib. In a randomized study of patients with the BRAF^{V600E} mutation, addition of the BRAF inhibitor, vemurafenib, to cetuximab-plus-irinotecan (VCI) versus cetuximab-plus-irinotecan (CI) resulted in an objective response (OR) rate of 16% v 4% and disease control rate of 67% v 22%, with a median progression-free survival of 4.3 v 2.0 months (HR 0.42, p=0.001)(2,3). In another subpopulation of mCRC patients with mismatch repair deficiency, immunotherapy has had striking benefit(4).

Evidence that cancer is a disorder of genes has transformed both diagnosis and treatment. Molecular profiling of colorectal tumors has better defined patients who may benefit from targeted therapy. In patients with KRAS wild-type (WT) tumors (exon 2, codons 12/13), treatment with cetuximab compared to supportive care significantly improved overall

survival (median 9.5 months *v* 4.8 months, hazard ratio 0.55; 95% confidence interval 0.41–0.74; *p*<0.001). In contrast, patients with KRAS-mutated tumors saw no survival benefit(5) from anti-EGFR targeted therapy. More recent studies have shown that expression levels of ligands and mutations in the RAS pathway predict lack of benefit to cetuximab(6,7). Even in responsive patients, serial analyses of tissue samples have shown that cancer cells adapt to pharmacologic pressure with expansion of pre-existing resistant subclones or by the acquisition of new resistance mutations(8). Experiments evaluating response of KRAS-WT tumors to anti-EGFR therapy in PDX models identified mutations in *ERBB2*, *EGFR*, *FGFR1*, *PDGFRA*, and *MAP2K1* as potential resistance mechanisms (9). Similarly, when cetuximab resistance was generated in CRC cell lines by gradually increasing drug exposure, 3/7 resistant cell lines acquired *ERBB2* amplification as demonstrated by fluorescence-in-situ hybridization(10).

HER2 amplification occurred in 4/11 (36%) PDX models from cetuximab-resistant patients with quadruple-WT genotype. In these PDX models, *HER2*-amplified mCRC was resistant to monotherapy with anti-EGFR agents, but responsive to therapy with lapatinib combined with either pertuzumab or cetuximab to block EGFR and *HER2*(11). In an independent PDX model from a quadruple-WT colorectal tumor, response to anti-EGFR monotherapy was transient but combination therapy blocking upstream and downstream pathways with cetuximab plus a MEK-inhibitor resulted in greater tumor reduction, which was maintained for the study duration(12).

In the HERACLES trial, 914 patients with KRAS exon 2 (codons 12/13) -WT mCRC were screened for *HER2*-amplification, with 48 (5%) patients identified. Twenty-seven patients with extensive prior treatment including anti-EGFR therapy were treated with the combination of trastuzumab-plus-lapatinib. Eight of 27 (30%) patients achieved an objective response and 12 (44%) had stable disease (SD). Nine of 27 patients showed no signs of progression for >6 months(13). Thus, in this small population of patients resistant to anti-EGFR therapy, *HER2* amplification was identified as an actionable target; indeed, a combination of anti-*HER2* targeted therapies did overcome resistance, resulting in prolonged disease control for some patients.

Neratinib is an irreversible pan-*ERBB* receptor TK inhibitor (*HER1* or *EFGR*, *HER2* and *HER4*), which has been shown to be more potent than lapatinib in cell-lines and xenografts. It has also been shown to be the most potent of the anti-*HER2* agents in a functional *HER2*-signaling assay (Celcuity)(14–16). Because EGFR is the primary driver in KRAS-WT mCRC, we speculated that dual blockade of *EGFR* and other *HER2*-family receptors would overcome resistance to cetuximab.

In this Phase Ib trial, we sought to determine safety and tolerability of neratinib-plus-cetuximab in selected patients who progressed during treatment with anti-EGFR antibodies with quadruple-WT mCRC, and the recommended phase II dose (RP2D) of neratinib-plus cetuximab that can be administered as a combination in Phase II. Secondary aims were to determine PFS in patients presenting with measurable metastatic disease by evaluating time-to-progression, and any differences in PFS between *HER2*-normal vs. *HER2*-amplified patients.

We determined the RP2D of neratinib when combined with cetuximab and preliminary efficacy with the combination was assessed in this population with quadruple-WT genotype and prior cetuximab treatment. Patients were not selected for *HER2* amplification. *HER2*-amplification status was evaluated retrospectively.

Methods

Patients

Enrollment occurred between February 2014–December 2015. Eligible patients, identified through the NSABP live tissue repository, were >18 years with ECOG PS=0–2, quadruple-WT (*KRAS*, *NRAS*, *BRAF*, *PIK3CA*) by central CLIA testing, and had measurable metastatic disease per Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Prior treatment with oxaliplatin and irinotecan as part of standard chemotherapy regimens and anti-EGFR therapy with either cetuximab or panitumumab was required. Patients were required to have archived tissue samples from their primary or treatment-naïve metastatic tumor. Upon enrollment, a research biopsy from a metastatic lesion was obtained. Acceptable laboratory parameters included an absolute neutrophil count (ANC) of 1000/mm³, platelet count of >100,000/mm³ and hemoglobin of ≥9.0 g/dL, a total bilirubin of ≤1.5 x ULN, and AST and ALT of ≤2.5 x ULN or if liver metastases ≤5 x ULN, and a serum creatinine of ≤1.5 x ULN.

Patients were excluded if they could not swallow oral medications, had symptomatic brain metastases, active hepatitis B or C with abnormal liver function tests, intrinsic lung disease causing dyspnea or symptomatic cardiac disease. The protocol was approved by the Institutional Review Boards of each institution and all patients provided written informed consent as required. The study was conducted according to good clinical practice and the Declaration of Helsinki. Authors had full control of all primary data.

Study design

NSABP FC-7 was designed as a multicenter, open-label study to evaluate the combination of weekly fixed-dose cetuximab plus daily neratinib with dose escalation using a 3+3 design in patients with treatment-refractory, mCRC with a quadruple-WT genotype (*KRAS* [exon 2], *NRAS*, *PIK3CA*, and *BRAF*). All patients had progressed on prior cetuximab or panitumumab. The primary study aim was to determine the safety profile of the two-drug combination and the RP2D of neratinib when added to cetuximab. Secondary endpoints were to determine the OR rate, clinical benefit rate (CBR), and investigate exploratory biomarkers.

Patients were recruited from our Molecular Profiling Registry (MPR-1), consisting of mCRC patients who consented to have their primary tumor tissue molecularly profiled. Tumors were initially pre-screened for hot-spot mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*, using our ColoCarta panel(17). All patients' tumors were CLIA-confirmed as quadruple-WT.

Treatment

Patients received concurrent therapy with a loading dose of cetuximab at 400mg/m² intravenously followed by weekly cetuximab at 250mg/m² plus neratinib, given orally once daily. Dose escalation of neratinib included four cohorts: 120mg, 160mg, 200mg, and 240mg. Standard pre-medications were given before each cetuximab administration. Because diarrhea is expected with neratinib, primary prophylaxis with loperamide beginning with the first dose was mandated. Primary prophylaxis during cycle-1 required patients to receive an initial 4mg dose of loperamide administered with the first dose of neratinib, followed by 4mg every six hours for 48 hours and to continue this regimen for grade 2 (G2) diarrhea. If after 48 hours, diarrhea was <G2, patients were instructed to take 2mg of loperamide every 4 hours while awake and 4mg at bedtime. During subsequent cycles, loperamide was titrated as needed. Prophylactic doxycycline 100mg PO BID was recommended beginning with the first dose of cetuximab to minimize rash(18,19).

Patients continued therapy until disease progression or discontinuation of study therapy due to patient withdrawal, physician discretion, or toxicity.

Safety assessment

Safety was assessed by physical examination, interim history, and laboratory assessment. Adverse event (AE) assessment occurred on days 1, 8, 15, and 22 of cycle-1 and on day 1 and 15 of each four-week cycle thereafter, up to 30 days following discontinuation of therapy. AE reporting was in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) v4.0. Patient safety and reported AEs were continuously monitored and reviewed by the NSABP medical review team on weekly teleconferences with designated NSABP personnel and participating site staff and investigators. DLT was determined during cycle-1. A DLT was the occurrence of 1 of the following during cycle-1:

- G3 diarrhea lasting ≥ 2 days on optimal medical therapy;
- G4 diarrhea of any duration or of any grade associated with fever or dehydration;
- G3/4 neutropenia associated with fever or G4 neutropenia lasting ≥ 7 days;
- G4 thrombocytopenia;
- G3/4 non-hematologic toxicity;
- Toxicity-related delay of ≥ 2 weeks.

Correlative Studies

We sought to explore molecular and genetic correlates for degree of benefit from neratinib-plus-cetuximab. We hypothesized that *HER2*-negative/normal colorectal tumors may acquire resistance to cetuximab by overexpressing *HER2* as one mechanism of anti-EGFR resistance. To test this hypothesis, *HER2* FISH assays were performed on required core biopsy tumor specimens obtained from an accessible metastatic lesion from cetuximab-resistant patients prior to study treatment. *HER2* amplification was also assessed in cfDNA from plasma obtained upon enrollment. Other correlative study hypotheses included: (1)

Changes in expression levels of RTKs, RTK ligands, and downstream signaling genes, may reflect response and acquired resistance to cetuximab monotherapy as well as sensitivity to combined neratinib-plus-cetuximab. To examine this hypothesis, we assessed differential expression of 2,560 genes using the HTG EdgeSeq technology and the Oncology Biomarker Panel; (2) The efficacy of neratinib-plus-cetuximab treatment may be determined by measuring changes in phosphorylation status and the up- or down-regulation of downstream signaling proteins and pathways. Using Reverse Phase Protein Micro-Arrays (RPPA), we compared enrollment biopsy samples with optional post-treatment samples obtained from consenting patients after receiving neratinib-plus-cetuximab, to evaluate signaling and acquired mechanisms of resistance to therapy with both targeted agents; and (3) Acquired/pre-existing mutations may predict response or resistance to neratinib-plus-cetuximab combination therapy. This hypothesis was evaluated by searching for mutations in cfDNA using a 74-gene Guardant panel(20).

Blood and tissue collections

Tissue requirements included treatment-naïve tissue samples from either the primary resection or a metastatic tumor (pre-anti-EGFR), and a second, paired tissue and blood sample upon enrollment (post-anti-EGFR). Blood, collected in EDTA tubes prior to treatment and following cycle-1, was analyzed for neratinib concentrations to determine pharmacokinetic parameters and for cfDNA in selected cases. In three cases, residual tumor tissue was collected after FC-7 study treatment.

Assessment of *HER2* amplification

Chromogenic *in situ* immunohistochemistry (CISH) was used to assess *HER2* copy number in enrollment biopsy tissues and matched treatment-naïve tumors and in one case, a metastatic biopsy collected post-progression on neratinib-plus-cetuximab. *HER2* CISH assessment was performed using the DAKO pharmDx™ reagents, following recommended protocols. The DAKO pharmDx™ (Carpinteria, CA), includes probes for *HER2/ERBB2* and the chromosome-17 centromere. Manual scoring of cells staining positive for each of these probes was performed by a board-certified pathologist and tissues scored as a ratio of *ERBB2*-positive cells to chromosome 17-positive cells. A ratio ≥ 2.0 is considered positive.

cfDNA Sequencing

cfDNA was isolated from plasma collected in EDTA tubes. *HER2* amplification was assessed with Next-Gen-Sequencing on an Illumina NextSeq 550 instrument in a CAP-accredited, CLIA-certified laboratory (Guardant Health). For CNV detection, probe-level unique-molecule coverage was normalized for overall unique-molecule throughput, probe efficiency, GC-content, and signal saturation. The coverage was then robustly summarized at the gene level. CNA determinations were based on training-set established gene-specific decision thresholds for both absolute copy number deviation from per-sample diploid baseline and deviation from the baseline variation of probe-level normalized signal in the context of background variation within each sample's own diploid baseline. The cut-off was binary using a copy number of >2.0. Mutations and gene-fusions were also scored using a 74-gene Guardant panel(20).

Reverse-phase protein micro-array analysis (RPPA)

Lysates were prepared from tumor cells isolated by macro-dissection of sections from FFPE slides, under conditions designed to preserve protein phosphorylation (21,22). Lysates from 49 samples were probed for 41 analytes using Reverse Phase Protein Micro-Array technology (Theranostics Health, Rockville, MD) (Supplementary Table ST1). The experimental and analytic methods are essentially as described in Pierobon (2017) and Pin (2014)(23,24).

HTG EdgeSeq

Gene-expression profiling was performed on the HTG-EdgeSeq platform (HTG Molecular Diagnostics Inc., Tucson, AZ). Crude lysates from macro-dissected tumor cells from FFPE tissue served as input, following manufacturer-recommended protocols. Differential gene expression of 2560 transcripts was assayed using the HTG Oncology Biomarker Panel.

PK analysis

Cycle-2 day 1 plasma concentrations of neratinib were quantitated with an LC-MS/MS assay fully validated per FDA guidance(25). Neratinib trough concentrations were extrapolated to the 24hr value assuming a 14.9hr half-life reported previously(26,27). Cetuximab total- and free-serum-concentrations were measured by ELISA (Somru BioScience Inc. National Research Council, Charlottetown, PE, Canada).

Statistical analyses

A standard 3+3 design was utilized to determine the safety, tolerability, and RP2D of neratinib combined with full-dose cetuximab. Dose escalation cohorts of neratinib were 120mg/d, 160 mg/d, 200mg/d, and 240 mg/d. The RP2D was the highest dose level at which <2/6 patients experienced a DLT in cycle-1. We did not reach the maximum tolerable dose defined as the highest dose with 2/6 patients experiencing a DLT, because dose escalation was stopped at the predetermined full dose of each drug as a single agent. Endpoint analyses were descriptive. An interim analysis was not conducted. Patients receiving any study therapy were considered evaluable for toxicity, whereas patients without toxicity in cycle-1 were considered evaluable for DLT only if they received at least 75% of neratinib doses in cycle-1. Patients who completed one cycle of therapy but discontinued treatment for any reason prior to their first scan (8 weeks) were considered to have progressive disease (PD).

For the pharmacokinetic analyses, correlations of trough levels with response and toxicity were explored with the Wilcoxon rank-sum test applied to trough levels in patients with and without response or toxicity(28). Statistical comparisons of exposures to neratinib, free cetuximab, and total cetuximab between patients with SD and PD, as best response, were assessed using the Wilcoxon rank-sum test.

Heatmaps were generated using the heatmap.2 package in R gplots. For comparisons between treatment-naïve and enrollment biopsies values were expressed as ratios ($\log_2(\text{post-anti-EGFR} / \text{pre-anti-EGFR})$) and are shown in the figures without scaling. For other figures, values were scaled across samples when drawing the heatmap.

Response assessment

Clinical activity was assessed by performing tumor measurements using RECIST v.1.1 criteria for all patients at baseline and after every two cycles (eight weeks) by documenting changes in the sum of the longest diameter (unidimensional measurement) of measurable target lesions and assessing presence or absence of non-measurable lesions. SD was defined as neither sufficient shrinkage to qualify for a partial response nor sufficient increase to qualify for PD, taking as reference the smallest sum of diameters since baseline. If PD occurred at any point, therapy was discontinued. Patients continued to be followed for 30 days after discontinuation of therapy or beginning alternative therapy.

Results

Patient characteristics

Results are reported using data collected up to the data lock on September 13, 2016. Eight institutions enrolled 22 patients who participated in the NSABP live tissue repository (MPR-1). All patients were quadruple WT. One patient withdrew consent before receiving study drugs. Characteristics of the 21 patients are shown in Table 1. Median age =62 (range 32–81). Median number of prior treatments was five. ECOG PS was 0 in 8 patients and 1 in 13.

Safety

Twenty-one patients who received at least one dose of neratinib-plus-cetuximab were evaluable for toxicity. Five patients were non-evaluable for DLT due to disease-related complications, one each at 120mg and 160mg neratinib dose levels, and three at 240mg. One patient (1/7) had a DLT at 240mg/day in cycle-1 with G3 diarrhea lasting >24 hours with dehydration requiring hospitalization and treatment discontinuation. Rapid resolution occurred upon stopping neratinib. One patient who received neratinib at 200mg/day had G3 diarrhea, which was self-limited and most likely related to an inter-current illness. There were no DLTs at 120 mg/d (0/3), 160 mg/d (0/3), or 200 mg/d (0/3). Overall, the combination of neratinib-plus-cetuximab was well tolerated in this heavily pretreated population. The RP2D are cetuximab 400 mg/kg loading dose followed by weekly cetuximab 250mg/kg intravenously and neratinib 240mg/d orally.

Treatment-related adverse events of special interest with neratinib-plus cetuximab included diarrhea, rash, and transaminase elevations. (Table 2).

Antitumor activity

Ten patients who received study drug did not complete two cycles of treatment and did not have tumor assessment by imaging. They included six patients who did not complete cycle-1: one patient who experienced a DLT, two who withdrew, and three who had disease-related complications during cycle-1. These six patients were considered non-evaluable for response assessment. The remaining four patients completed cycle-1 but had early clinical progression, did not complete cycle-2, and did not have week-8 imaging. They are included in the response assessment. Of the eleven patients having 1 follow-up scan, four had PD as best response and seven had SD four of whom were *HER2*-amplified either in primary

tumor or enrollment biopsy. No patient achieved a partial or complete response by RECIST 1.1. Duration of SD ranged from 14–23 weeks. (Table 3).

Pharmacokinetic analysis

Fifteen patients had evaluable steady-state neratinib plasma trough samples collected between 15–30h post-dose. All concentrations were above the assay lower-limit of quantitation of 2ng/mL. Incurred sample reanalyses, successfully performed with all samples and repeats, were all within 20% (29). Calculation of trough concentrations at 24hr (C_{24}) resulted in median absolute concentration adjustments of 6.8% (range 33–30%). The mean C_{24} was 19.8 ng/mL with a 60 CV%, with large variability of exposure at any given dose (Fig. 1a). Dose normalization of C_{24} resulted in a mean of 0.124 ng/mL/mg with a 77 CV%. The one patient who experienced transient G3 diarrhea on 160mg neratinib had a dose reduction to 120mg before the cycle-2, day 1 PK sample was drawn. With dose-normalized C_{24} of 0.34 ng/mL/mg at the time of G3 diarrhea, the corresponding absolute value would have been 54.3 ng/mL, the highest of all C_{24} values observed.

Cetuximab concentrations were measured in duplicate (total cetuximab median difference 4.1%, range 0.8–7.8%; free cetuximab median difference 2.2%, range 0.2–28%), and average values were used. At the beginning of cycle-2 total cetuximab concentrations averaged 93.2 μ g/mL (SD 50.5) and free cetuximab concentrations were on average 95.3 μ g/mL (SD 96.0) with free cetuximab representing a median of 86.6% (range 5.4–168) of total (Fig. 1b).

Exposures to neratinib, free cetuximab, and total cetuximab did not significantly vary between patients with SD and PD, as best response (Fig. 1c).

Correlative science studies

Altered *HER2* signaling may be an important factor in determining response to anti-EGFR therapy. We assessed the *HER2* RNA expression, total protein, and phosphorylation levels, in tissues that were treatment naïve (n=21), and in enrollment biopsy tissues collected (post-anti-EGFR) (n=16) and post-neratinib (n=3).

HER2 amplification in tissues and blood

HER2 amplification in treatment-naïve tumor tissue and in the enrollment biopsy (post-anti-EGFR treatment) was evaluated using CISH (Table 3; Supplementary Fig. SF1). Of 21 treatment-naïve tissues evaluable for *HER2* amplification, two (9.5%) showed pre-existing *HER2* amplification. Four of 16 (25%) patients with evaluable biopsies collected upon enrollment, post anti-EGFR therapy, showed *HER2* amplification. Six patients did not have biopsies with sufficient tumor content to evaluate *HER2*-amplification status. In one of these cases (FC-476, Table 3), in addition to the treatment-naïve and enrollment samples, a third sample, collected after treatment with neratinib-plus-cetuximab, was also evaluated, revealing *HER2* amplification.

Assessment of *HER2* amplification in cfDNA

Next, we sought to determine whether results from *HER2* amplification assayed in tissue by CISH correlated with amplification assessed in blood using the Guardant cfDNA assay. Our comparison was limited to specimens collected upon enrollment (post-anti-EGFR therapy), because these tissue and blood samples were obtained simultaneously. For eight patients with paired samples of tissue and blood, there was concordance in the *HER2*-amplification status between tissue and blood for six (75%) (Table 3). In one of the two discordant cases, the tissue-based CISH assay indicated no amplification, whereas the cfDNA assay indicated amplification (FC-7-335). Notably, this patient had a SD response. This type of discordance has been reported elsewhere(30). In a second case (FC-7-403), discordance went in the opposite direction - i.e., the tissue biopsy was positive for *HER2* amplification by CISH, whereas the cfDNA result was negative. One other sample (FC-7-645) in which the enrollment biopsy had inadequate tumor content showed markedly high amplification (22X) in the treatment-naïve tissue and blood samples (Table 3). This patient had SD post-treatment.

Assessment of cfDNA Mutations

Using a 74-gene Guardant panel, we assessed the mutation profile in cfDNA from 11 patients for whom plasma was available(20). Although alterations in 30 genes were uncovered at mutation allele frequencies ranging from ~70% down to the limits of detection, no consistent patterns were seen that correlated with outcomes (Supplementary Fig. SF2; Supplementary Table ST2). For example, no mutations were detected in the cfDNA from patient FC-7-327, whereas patient FC-7-335 showed 15 alterations, including *PIK3CA* (14% MAF), *BRCA2* (8% MAF), *KRAS* (4% MAF), and 12 other alterations at MAFs close to the limit of detection. However, both patients had SD. Mutations in *Tp53* and *APC*, occurring at high MAFs, were found in all three groups - SD, PD, and non-evaluable patients. Other mutation profiles are discussed in the supplement.

Gene expression

We hypothesized that elevated expression of the *ERBB* receptors or their ligands may confer resistance to cetuximab but maintain sensitivity to other combinations of anti-EGFR and anti-*HER2* agents. To test this, we examined the expression levels of the *ERBB* receptors/ligands in the enrollment biopsy samples by targeted gene expression analysis using HTG-EdgeSeq technology and the Oncology Biomarker panel. Examination of *ERBB1*, 2, 3, and 4 and HER-family ligands *AREG*, *EREG*, and *NRG1*, 2, and 4 revealed that, based on a z-score analysis, all 16 available cases showed elevated expression of one or more of these genes (Fig. 2a). A majority of patients (14/16) (87.5%) showed relative upregulation of 2 members of the HER-family receptors or ligands between treatment-naïve and post-anti-EGFR tissues (Fig. 2b). Such a result might indicate that these patients would be responsive to a pan-HER inhibitor such as neratinib. However, clinical outcomes in this study did not correlate with these gene expression changes.

In the context of this trial, it is relevant to ask whether anti-EGFR therapy resulted in the upregulation of HER-family members that would not be inhibited by anti-EGFR therapy but may be inhibited by neratinib, which is a pan-HER inhibitor. Because we collected both

treatment-naïve and enrollment biopsies, we were able to use paired samples to compare gene-expression differences within individual patient samples after exposure to anti-EGFR therapy. A majority of cases showed overexpression of *ERBB3* (13/15) (87%) following anti-EGFR treatment. Expression of *ERBB2*, *ERBB4*, and *EGFR*, fell into two classes – one showing upregulation after anti-EGFR treatment, the other showing under-expression (Fig. 2b).

RPPA analysis

Proteins constitute the predominant drug targets and are the primary mediators of oncogenic signaling pathways. Therefore, we used RPPAs to examine total protein and protein phosphorylation of important signaling proteins in treatment-naïve and enrollment biopsy samples. A set of receptor TKs, downstream signaling effectors, as well as proliferation and apoptosis markers were assayed, for a total of 41 analytes, including total protein and specific phosphorylation sites (Supplementary Table ST1; Fig. 3a,b,c). When all the analytes are considered together (Fig. 3a), the enrollment biopsy samples fall into two main classes: one showing marked activation of a majority of the targets examined, and another showing downregulation of signaling.

Fig. 3b shows a heatmap of the RPPA assay for the HER-family signaling proteins. Matched primary tissue and enrollment biopsy samples obtained after anti-EGFR therapy were assayed. The heatmap records the changes in the analytes post-anti-EGFR treatment. The 16 cases examined fell into two clusters based on changes in HER-family proteins post-anti-EGFR therapy – one group showed marked activation of HER-family genes, shown in red, whereas the other showed a downregulation of HER signaling, shown in green. Upregulation of total EGFR was seen in 9/16 samples. In 6/9 cases, activation of *ERBB3* and/or *ERBB4* phosphorylation at specific sites accompanied elevated total EGFR. Levels of total *ERBB2* protein correlated with DNA-level amplification of *HER2* (yellow highlighting, Fig. 3b).

Fig. 3c shows the top signals observed in the RPPA assay, for downstream signaling effectors beyond the HER family. Elevated phosphorylation is observed for AKT, GSK3b, and MEK1 in baseline tissues (post-anti-EGFR therapy, upon enrollment), indicating signaling heterogeneity in the study population upon entry. As shown in Fig. 3c, phosphorylation of AKT (S473) is upregulated, to varying extent, in all 16 pairs, MEK1 (S298) phosphorylation is upregulated in 10/16 pairs, GSK3b (S219) in 13/16 pairs, and ERK1.2 (T202, Y204) in 12/16 pairs. In the case of AKT and ERK1.2, an increase in the RNA is also seen as assayed by HTG-EdgeSeq (data not shown).

Although not among the top six signals observed, ERK1.2 total protein is included because downregulation of total ERK1.2 accompanied by increased phosphorylation (ERK 1.2 T202, Y204), indicates high activation.

Examination of post-neratinib-plus-cetuximab tissues

Next, we compared gene-expression profiles in three cases for which paired tissue samples were available from both enrollment biopsies (post-anti-EGFR) and post-neratinib-plus-cetuximab treatment (Supplementary Fig. SF3). All three pairs showed an upregulation of HER-family-ligands *NRG1*, 2, 3, and 4, post-neratinib-plus-cetuximab, raising the

possibility that *NRG* genes may mediate resistance to neratinib. Such a change was not observed in similar comparisons between pre- and post-anti-EGFR samples.

Discussion

Although the combination of neratinib-plus-cetuximab was well tolerated, we did not observe any patients achieving enough tumor shrinkage to meet RECIST 1.1 criteria for an objective response (OR). Dose-escalation progressed through four pre-specified cohorts with only one patient experiencing dose-limiting G3 diarrhea at 240mg/day of neratinib. In contrast to our study, in the HERACLES trial (trastuzumab-plus-lapatinib in *HER2*-amplified patients) ORs were seen in 8/27 patients. Preclinical studies with quadruple-WT, cetuximab-resistant PDX models demonstrated that dual EGFR-HER-family pathways blockaded with trastuzumab- or pertuzumab-plus-lapatinib were more effective than either drug as a single agent(11). One might speculate that the *HER2* pathway may act as an oncogenic driver and also mediate resistance to anti-EGFR therapy at one or more levels: the level of *HER2* expression, the extent of heterodimerization of *HER2:HER3*, or the increased expression of ligands; thus, a more complete block of this pathway(31,32) may be required for therapeutic efficacy.

Our main hypothesis for the correlative science studies was that a subset of CRC tumors develops acquired resistance to cetuximab treatment by elevated *HER2* signaling. *HER2* overexpression or amplification following treatment with cetuximab has been previously described as a mechanism of acquired resistance(10). Frequency of *HER2* amplification has been reported as 2–3% in unselected cases, 13% in *KRAS*-WT, cetuximab-resistant populations, and as high as 36% in quadruple-WT xenopatient(11).

HER2 amplification occurred in 2/21 (9.5%) treatment-naïve tissues and in at least four enrollment biopsy tissues (4/16, 25%). Paired tissue and blood samples were available in eight cases and analyzed for *HER2* amplification in tissue by CISH and in plasma by cfDNA, with 75% concordance. An additional sample (FC7–645, Table 3) was concordant between primary tissue and blood collected upon entry (enrollment biopsy sample was inadequate), thus, overall concordance=87%, consistent with earlier reports(33–35). Although limited by sample size, these results support earlier observations that anti-EGFR treatment may drive *HER2* amplification and the development of treatment resistance. Discordance in *HER2* amplification between tissue and cfDNA was observed in both directions – in FC-7–335, amplification was observed in cfDNA but not tissues; this may be explained by the sensitivity and systemic nature of liquid biopsy assays, which measure cfDNA originating from any site, unlike single, directed tissue biopsies. FC-7–403 was amplified in the tissue, but not in cfDNA; this may be explained by variations in tumor burden, treatment response, tumor cell shedding, vascularity, and cfDNA release into the bloodstream as reported elsewhere(36,37).

The ability to identify mCRC patients with *HER2* amplification in a blood sample is not only less invasive but may also be more representative of tumor heterogeneity(37,38). Despite detection of *HER2* amplification in six patients in either tissue or blood samples, the best response in these patients was SD. Because *HER2* amplification is one mechanism of

anti-EGFR resistance, which appears to occur under the pressure of therapy, we speculate that it may be preferable to treat KRAS-WT patients with dual therapy (anti-EGFR and anti-*HER2* therapy) even in the absence of identified *HER2* amplification, in an effort to prevent or delay emergence of resistance(39). Several examples from PDX models exist, supporting this approach. In a PDX population of quadruple-WT tumors without prior anti-EGFR therapy, monotherapy with cetuximab resulted in a high frequency of responses, mostly SD. Dual therapy with cetuximab-plus-lapatinib in the regrown models with SD resulted in 5/21 having a major response, (75% decrease in tumor-volume)(40), suggesting that the combination provides, in principle, a more complete upstream blockade of driver signals. In an additional experiment with quadruple-WT PDX models, treatment with cetuximab resulted in initial response followed by resistance and tumor growth. Treatment with the MEK inhibitor, pimasertib, as a single agent was ineffective, but the cetuximab-plus-pimasertib combination resulted in dramatic and long-lived tumor shrinkage. Biochemical studies showed that resistance to EGFR blockade is most frequently accompanied by activation of MEK and ERK and that vertical suppression of the EGFR pathway may prevent or delay resistance(40).

In RPPA studies, the observations in Fig. 3a show samples falling into two main classes: one showing marked activation of a majority of the targets examined, and another showing downregulated signaling. These two post-anti-EGFR groups enrolling into FC-7 may, therefore, constitute two disparate populations, requiring different, although not necessarily mutually exclusive, treatment approaches. 6/16 cases (36%) showed elevated signaling of at least one HER family member. Increased phosphorylation was observed for *ERBB2*, -3, and -4, indicating elevated signaling. The levels of total *ERBB2* protein correlated with the DNA-level amplification of *HER2* (yellow highlighting, Fig. 3b). Elevated phosphorylation of *ERBB* family members indicates that *HER2* amplification correlated with activation of the *ERBB2* pathway. Despite limited sample numbers, our results from the gene-expression and the (phospho-) protein studies both indicate that although anti-EGFR therapy was effective in limiting EGFR signaling, it was not therapeutically efficacious by RECIST criteria, suggesting that additional anti-*HER2* therapy combinations may be required to block this pathway. Downregulation of total ERK1.2 protein accompanied by increased phosphorylation (ERK 1.2 T202, Y204) suggests that even though the protein level (perhaps driven transcriptionally) is low, the activation, driven post-translationally, is high. This increased phosphorylation, and consequent activation of ERK1.2, leads to the speculation that ERK1.2 may present a potential target for combination therapy.

Probing a set of 2,560 cancer-related genes, we compared gene-expression in samples collected upon enrollment with treatment-naïve tissues. Elevated expression of one or more HER-family receptors/ligands was seen in a majority of patients (Fig. 2a,2b). Increased expression of *ERBB3* and other HER-family receptors/ligands indicated increased *ERBB* signaling (Fig. 2b), confirming the rationale that such tumors could be sensitive to a more comprehensive blockade of the HER-pathway, with combinations including agents such as neratinib. However, clinical outcomes in FC-7 did not correlate with gene expression changes, suggesting that additional anti-*HER2* therapy combinations may be required to effectively block this pathway.

The three cases for which residual tumors could be compared to baseline samples show elevated expression of *ERBB* ligands *NRG1*, 2, 3, and 4 post-neratinib (Supplementary Fig. SF3). When taken together with the clinical outcomes, this intriguing observation suggests that, in tumors driven by increased *NRG*-ligand expression, more potent dual anti-*HER2* blockade with combinations involving neratinib, a highly specific pan-*ERBB* inhibitor, plus trastuzumab, may be more effective than cetuximab, which targets the EGFR receptor. A clinical trial, FC-11 ([NCT03457896](#)), is currently underway testing this combination.

Our study highlights the complexity of variations in the proteogenomic status of patients as they move through sequential lines of therapy. Genome-scale assays may be more informative in evaluating therapy resistance and sensitivity than focused panels such as the ones used in this study for RNA, phosphoprotein, and mutational analyses. Recent advances in microscaled proteogenomics may facilitate such analyses even with limited tissue(41). However, although evolving technologies allow high assay throughput, for the foreseeable future, the analysis, interpretation, and clinical decision-making based on the data still need to be approached as an n-of-one effort for each individual patient.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors acknowledge the contributions of Christine I. Rudock, Publications and Graphics Specialist and Wendy L. Rea, BA, Editorial Associate, both of whom are employees of NSABP. They were not compensated beyond their normal salaries for this work.

Support: Puma Biotechnology, Inc; This project used the UPMC Hillman Cancer Center Cancer Pharmacokinetics and Pharmacodynamics Facility (CPPF), and was supported in part by awards NCI P30 CA047904 and R50 CA211241; and NSABP Foundation, Inc.

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Previous, related works:

- Kiesel BF, Parise RA, Wong A, Keyvanjah K, Jacobs S, Beumer JH. LC-MS/MS assay for the quantitation of the tyrosine kinase inhibitor neratinib in human plasma. *J Pharm Biomed Anal* 2017;134:130–36. [PubMed: 27907855]
- Jacobs SA, Lee JJ, George TJ, Wade JL III, Stella PJ, Wang D, Sama AR, Buyse ME, Kanyuch JA, Srinivasan A, Pogue-Geile KL, Kim SR, Wolmark N, Allegra CJ. NSABP FC-7: A phase Ib study

evaluating neratinib and cetuximab (Cmab) in patients with quadruple wild-type (KRAS/NRAS/BRAF/PI3KCA wt) metastatic colorectal cancer resistant to Cmab. Mol Cancer Ther/AACR Molecular Targets 2015. Abstr C46.

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Translational Relevance

Molecular profiling of the *ERBB2* signaling pathway suggests that patients enrolled in FC-7 after receiving anti-EGFR treatment fall into distinct clusters. However, these clusters did not correlate with response to trial treatment. Our observations argue that (a) each group of patients is molecularly distinct and perhaps ought to be treated differently, (b) more extensive, pathway-agnostic profiling may be necessary to direct therapy - - i.e., the elevated gene-expression of the *ERBB* family ligands post-FC-7, as compared to enrollment biopsy tissues, suggests that it may be a mechanism of resistance, and (c) ctDNA analysis may be an effective, non-invasive tool to assess *ERBB2* gene amplification.

Figure 1.

A Neratinib trough concentrations on cycle-2, day-1 at 24h (C₂₄) after dosing, plotted as a function of neratinib daily dose. **B** Total and free trough serum concentrations of cetuximab on cycle-2, day-1. **C** Cycle-2, day-1 trough concentrations in patients with progressive disease (PD) or stable disease (SD) as best response. Neratinib (C₂₄, ○, Wilcoxon 2-tailed exact rank P=0.694), free cetuximab concentration (◇, Wilcoxon 2-tailed exact rank P=0.779), and total cetuximab concentration (△, Wilcoxon 2-tailed exact rank P=0.463), with medians displayed as horizontal bars.

Figure 2.

A Expression of *ERBB* receptor and ligand family members as assayed at the RNA level using HTG EdgeSeq upon enrollment into NSABP FC-7. The heatmap was generated using the normalized(rpm value) HTG data. It shows the expression of HER family transcripts across FC-7 enrollment samples. The expression was normalized across all samples (i.e., column-wise). Red indicates that a transcript has a relatively higher expression in certain samples compared with other samples. **B** Comparison of gene-expression profiles between the treatment-naïve (pre-anti-EGFR) primary tumor and metastatic enrollment biopsies (post-anti-EGFR) expressed as a ratio ($= \log_2(\text{post-anti-EGFR}/\text{pre-anti-EGFR})$). This heatmap was generated using normalized(rpm value) HTG EdgeSeq data. Red indicates higher expression after anti-EGFR therapy, upon FC-7 enrollment.

Figure 3.

A Reverse-phase protein micro-array analysis (RPPA) of post-anti-EGFR, metastatic biopsy samples collected upon enrollment into FC-7. Total protein and specific protein phosphorylation sites were assayed. A total of 41 analytes, as indicated on the x-axis, were examined, covering pathways implicated in HER2 signaling. **B** Comparison of total protein and protein-phosphorylation status, as assayed by RPPA, between the treatment-naïve (pre-anti-EGFR) primary tumor and metastatic enrollment biopsies (post-anti-EGFR), expressed as a ratio($\log_2(\text{post-anti-EGFR}/\text{pre-anti-EGFR})$). Red indicates higher levels of total protein or elevated protein-phosphorylation after anti-EGFR therapy, upon enrollment into FC-7.

The two far-left columns indicate HER2 amplification status, in primary (treatment-naïve) and metastatic biopsies collected upon enrollment into FC-7 respectively. Non-amplified (purple), amplified (yellow). **C** Comparison between the treatment-naïve (pre-anti-EGFR) primary tumor and metastatic biopsies (post-anti-EGFR) collected upon enrollment into FC-7 for the top RPPA signals, focusing on downstream effectors of HER2 signaling. Values expressed as a ratio ($\log_2(\text{post-anti-EGFR/pre-anti-EGFR})$) were used to produce the heatmap.

Table 1.

Patient characteristics - NSABP FC-7

Patient Characteristics	N=21
Age (years)	62 median (32–81 range)
Sex	
Male	13(62%)
Female	8 (38%)
ECOG Performance Status	
0	8 (38%)
1	13 (62%)
<i>HER2</i> expression at baseline by CISH	
Non-amplified	11(52%)
Amplified	4 (19%)
Unknown	6 (29%)
Previous lines of therapy	2–10
Median	4
Diameter of largest target lesion (mm)	
Mean	56.7
Median	48.0
Range	13–132

Table 2.

Grade 2–4 adverse events attributable to study therapy in 21 patients - NSABP FC-7

CTCAE v4.0	No. of patients with Grade 2 n (%)	No. of patients with Grade 3 n (%)	No. of patients with Grade 4 n (%)
No. of patients with 1 AE*	19 (90%)	14 (67%)	3 (14%)
Diarrhea	4 (19%)	2 (9%)	0
Nausea	4 (19%)	0	0
Vomiting	0	1 (5%)	0
Dehydration	2 (9%)	3 (14%)	0
Electrolyte imbalance	0	3 (14%)	0
Elevated transaminases	3 (14%)	1 (5%)	1 (5%)
Thrombocytopenia	1 (5%)	0	0
Fatigue	5 (24%)	3 (14%)	0
Rash	4 (19%)	1(5%)	0
Sepsis	0	0	2 (10%)

CTCAE indicates National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE) v4.0.

* A single patient may experience multiple grades of AEs.

Table 3.*HER2* amplification status in treatment-naïve and post-anti EGFR samples - NSABP FC-7

Sample	Treatment-naïve		Post-anti-EGFR	
	CISH (Tissue)	CISH (Tissue)	Guardant CNV (Blood)	Response
FC-7-046	NA	NA	Not Amplified	SD
FC-7-084	1.0	1.3	NA	NE
FC-7-295	1.0	1.6	NA	PD
FC-7-327	1.0	1.3	Uninformative	SD
FC-7-335	2.5	1.5	2.66	SD
FC-7-365	1.1	NA	NA	NE
FC-7-403	1.0	3.7	Not Amplified	SD
FC-7-408	1.0	2.1	NA	SD
FC-7-430	1.0	1.8	Not Amplified	PD
FC-7-431	1.0	NA	NA	NE
FC-7-476 *	1.0	1.0	NA	PD
FC-7-476 †	1.0	4.4	NA	
FC-7-531	1.2	1.7	NA	NE
FC-7-568	1.0	NA	NA	PD
FC-7-570	1.2	2.7	2.48	SD
FC-7-599	1.0	1.3	Not Amplified	NE
FC-7-645	>10 clusters ‡	NA	21.86	SD
FC-7-833	1.0	2.0	2.20	NE
FC-7-864	1.3	1.1	Not Amplified	PD
FC-7-905	1.0	NA	NA	PD
FC-7-934	1.0	1.1	NA	No tx
FC-7-961	1.0	1.5	NA	PD
FC-7-979	1.0	1.5	Not Amplified	PD
FC-7-xxx	NA	NA	NA	NE
Total % amp	2/21=9.5%	4/16=25%	Concordance=75% §	

Blood samples were collected upon enrollment into FC-7. All tissues samples are baseline enrollment biopsies with the exception of FC-7-476. Patient FC-476 samples were collected not only upon enrollment (as for all other biopsies), but also post-progression, as indicated:

* Enrollment biopsy.

† Post-progression.

‡ Indicates a cluster of positive signal (i.e., cells staining positive for amplification) in which spots were so numerous that they could not be individually resolved. A cluster >10 indicates a ratio >2 according to the scoring criteria recommended by the manufacturer of the CISH reagents (DAKO pharmDx™).

§ Concordance: tissue vs blood.

NA indicates “samples not available.” FC-7-645 scored as amplified in the treatment-naïve tissue sample and in the cfDNA liquid-biopsy, however, the biopsy collected upon enrollment did not have sufficient tumor content to be evaluable by CISH.

Amp indicates amplification.

SD indicates stable disease.

NE indicates non-evaluable.

PD indicates progressive disease.

Samples with amplification >2.0 threshold are indicated in red.

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