ABSTRACT

The Cancer Genome Atlas project identified HER2 somatic mutations and gene amplification in 7% of patients with colorectal cancer. Introduction of the HER2 mutations S310F, L755S, V777L, V842I, and L866M into colon epithelial cells increased signaling pathways and anchorage-independent cell growth, indicating that they are activating mutations. Introduction of these HER2 activating mutations into colorectal cancer cell lines produced resistance to cetuximab and panitumumab by sustaining MAPK phosphorylation. HER2 mutants are potently inhibited by low nanomolar doses of the irreversible tyrosine kinase inhibitors neratinib and afatinib. HER2 gene sequencing of 48 cetuximab-resistant, quadruple (KRAS, NRAS, BRAF, and PIK3CA) wild-type (WT) colorectal cancer patient-derived xenografts (PDX) identified 4 PDXs with HER2 mutations. HER2-targeted therapies were tested on two PDXs. Treatment with a single HER2-targeted drug (trastuzumab, neratinib, or lapatinib) delayed tumor growth, but dual HER2-targeted therapy with trastuzumab plus tyrosine kinase inhibitors produced regression of these HER2-mutated PDXs.

SIGNIFICANCE: HER2 activating mutations cause EGFR antibody resistance in colorectal cell lines, and PDXs with HER2 mutations show durable tumor regression when treated with dual HER2-targeted therapy. These data provide a strong preclinical rationale for clinical trials targeting HER2 activating mutations in metastatic colorectal cancer.

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See related commentary by Pectasides and Bass, p. 799.
HER2 activating mutations are targets in colorectal cancer. The majority of these mutations were shown to be activating based on their ability to increase intracellular signaling, induce oncogenic transformation, and accelerate xenograft tumor growth (6–8). HER2 activating mutations tend to fall in several hotspots (residues 309–310 in the extracellular domain and residues 755–781 and 842 in the kinase domain), and they are responsive to HER2 tyrosine kinase inhibitors (6, 7). These preclinical data have led to two multi-institutional, phase II clinical trials that will screen patients with metastatic breast cancer for HER2 mutations and treat the mutation-positive patients with the second-generation HER2/EGFR tyrosine kinase inhibitor neratinib (9, 10). Further, phase I and II clinical trials for HER2 mutations in NSCLC demonstrate the clinical efficacy of combining neratinib with the mTOR inhibitor temsirolimus (11, 12).

In this study, we determined the effect of HER2 somatic mutations in colorectal cancer. The HER2 mutations found in colorectal cancer are similar to those found in breast cancer. We demonstrate that these HER2 mutations cause oncogenic transformation of colon epithelial cells and produce resistance to cetuximab and panitumumab in two colorectal cancer cell lines. We identified HER2 activating mutations in colorectal cancer patient-derived xenografts (PDX) and demonstrated that dual HER2-targeted therapy causes tumor regression. These data form a strong preclinical rationale for clinical trials targeting HER2 activating mutations in patients with metastatic colorectal cancer.

RESULTS

HER2 Mutations Identified from Patients with Colorectal Cancer Cause Oncogenic Transformation of Colon Epithelial Cells

The TCGA colorectal cancer project identified HER2 alterations in 7% (14/212) of cases. Six cases had HER2 somatic mutations, five had HER2 gene amplification, and three had both HER2 mutations and HER2 amplification (Fig. 1A). Concurrent mutation and amplification have been described for other oncogenes, including RAS and EGFR (13). In addition to the TCGA colorectal cancer study, a recent sequencing study on patients with colorectal cancer performed at Memorial Sloan Kettering Cancer Center identified 3 more HER2-mutated cases (14). The HER2 mutations reported in both studies are combined in Fig. 1B, and several of these mutations are identical to HER2 mutations found in patients with breast cancer, including the kinase domain mutations V842I, V777L, and L755S, and the extracellular domain mutation S310F. Unlike the HER2 kinase domain mutations found in NSCLC, no HER2 kinase domain in-frame insertions/deletions were reported in these two colorectal cancer sequencing studies (8, 15). The HER2 mutations V777L and V842I were seen in multiple patients, with 2 cases with HER2V777L and 4 cases with HER2V842I identified (Fig. 1B). Half (6/12) of these HER2-mutated colorectal cancer cases are KRAS wild-type (WT), and one third (4/12) of these cases are quadruple WT (KRAS, NRAS, BRAF, PIK3CA; Supplementary Table S1). The co-occurring KRAS mutations included 4 cases of codon 12/13 mutations and 2 cases of exon 4 mutations (KRASQ61H and KRASG12D). The co-occurring BRAF mutations in HER2-mutated cases included one case with BRAFV600E (TCGA-AA-3947) and one case with BRAFV599L (TCGA-AG-A002). The BRAFV247L mutation is located in the C1 domain of BRAF; it has not been reported in any other sample in the cBioPortal and Catalogue of Somatic Mutations in Cancer (COSMIC) databases to date, and, to our knowledge, it has not been functionally characterized to date (16, 17). HER2-mutated colorectal cancers occurred in both the right and left sides of the colon as well as in the rectum (Supplementary Table S1). The microsatellite stability (MSS) or instability (MSI) status of these cancers was reported, and 83% (10/12) were MSS (Supplementary Table S1). MSS colorectal cancers have a worse prognosis than MSI colorectal cancers and show clinical benefit from 5-fluorouracil-containing adjuvant chemotherapy (18, 19).

To analyze the effects of HER2 mutations, we introduced these mutations into Immortalized Mouse Colon Epithelial (IMCE) cells and measured their effect on cell signaling and anchorage-independent growth. IMCE cells are nontransformed colon epithelial cells and can be transformed by the introduction of oncogenes (20, 21). We stably introduced WT or four HER2 mutations into IMCE cells using a retroviral vector (Fig. 1C). All four HER2 mutations increased HER2 signaling pathways, with increased HER2, MAPK, and AKT phosphorylation seen relative to the HER2WT transduced cells (Fig. 1C). Phosphorylation of the immediate HER2 substrate, phospholipase γ1 (PLCγ1), was greatest in the HER2V777L cells, but was also increased in the other HER2 mutations. The effect of HER2 mutations on soft-agar colony formation was also tested. All HER2 mutations dramatically increased the number of colonies formed in soft agar, demonstrating enhanced anchorage-independent growth (Fig. 1D; Supplementary Fig. S1A). Interestingly, in our prior study that used an MCF10A immortalized breast epithelial cell line, the HER2V599L mutation did not increase soft-agar colony formation or alter colony morphology in Matrigel (6). This difference in soft-agar colony formation between MCF10A–HER2V599L and IMCE–HER2V599L likely is due to subtle differences between the two cell lines. The effect of trastuzumab and neratinib on soft-agar colony formation was also tested (Fig. 1D). Trastuzumab produced statistically significant reductions in colony formation with L755S, L866M, and S310F mutations, whereas neratinib prevented colony formation with all of the mutations tested here. The effect of neratinib on IMCE–KRAS cells was also tested. IMCE–KRAS cells are relatively resistant to neratinib, with no effect on soft-agar colony formation at 50 to 100 nmol/L neratinib, whereas IMCE–HER2V599L cells show an IC50 of approximately 10 nmol/L neratinib in the soft-agar colony-formation assay (Supplementary Fig. S1B).

HER2 Mutations Cause Resistance to EGFR Monoclonal Antibodies in Colorectal Cancer Cell Lines

To determine whether HER2 mutations cause resistance to the EGFR monoclonal antibodies cetuximab and panitumumab, we first introduced the HER2V599L mutation into the...
cetuximab-sensitive colorectal cell line DiFi using a retroviral vector (Fig. 2A and B). We initially focused on the HER2 V842I mutation because it was the most prevalent mutation identified in colorectal cancer cases by TCGA (3). Introduction of HER2 WT into DiFi cells caused a modest (2–4-fold) change in the cells’ sensitivity to cetuximab and panitumumab (red curves, Fig. 2A and B). In contrast, introduction of the V842I mutation caused a 40- to 100-fold shift in the IC₅₀ values (blue curves, Fig. 2A and B). NCI-H508 is another cetuximab-sensitive, colorectal cancer cell line, and they are more readily transduced with retroviral vectors than DiFi cells. Five HER2 mutations were tested in NCI-H508 cells, and all mutations produced resistance to cetuximab and panitumumab (Fig. 2C and D). Western blots of the EGFR–HER2 signaling pathways suggest the mechanism of EGFR antibody drug resistance. Cetuximab treatment of parental DiFi or NCI-H508 cells reduced MAPK and EGFR phosphorylation (Fig. 2E and F).

Introduction of HER2 mutations into these cells increased MAPK and EGFR phosphorylation, and this was sustained even in the presence of cetuximab.

**HER2 Mutants Are Highly Sensitive to the Irreversible HER2/EGFR Tyrosine Kinase Inhibitors Neratinib and Afatinib**

Prior studies in breast cancer and NSCLC showed that HER2 mutations can be potently inhibited by nanomolar doses of neratinib or afatinib, which are irreversible HER2/EGFR tyrosine kinase inhibitors (6, 7). We therefore tested the effect of neratinib and afatinib on DiFi and NCI-H508 cells transduced with HER2 WT or HER2 mutations. Neratinib and afatinib inhibited the growth of parental DiFi cells, DiFi–HER2 WT cells, and DiFi–HER2 V842I cells, with IC₅₀ values ranging from 2 to 5 nmol/L (Fig. 3A and B). Similarly, growth of parental NCI-H508 cells and cells transduced with WT or

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**Figure 1.** HER2 mutations identified by colorectal cancer genome sequencing studies increase cell signaling and anchorage-independent growth in a colonic epithelial cell line. A, HER2 alterations identified by the TCGA colorectal cancer project. B, HER2 somatic mutations observed in 12 patients with colorectal cancer are shown. Red circles, TCGA cases with HER2 gene amplification; blue circles, TCGA cases that do not have HER2 gene amplification; green circles, cases from Brannon et al. (14), and gene amplification is not reported on these cases. One TCGA case had concurrent V842I plus V777L mutations along with gene amplification. FU, Furin-like domains; TM, transmembrane region. C, IMCE cells were retrovirally transduced with HER2 WT or mutants. Total lysates (8–10 μg) were analyzed by Western blot. D, IMCE HER2 WT or HER2 mutants were seeded in soft agar in duplicate, treated with trastuzumab (100 μg/mL) or neratinib (500 nmol/L), allowed to grow for 12 days, and stained with crystal violet. Photographs of the stained wells are shown in Supplementary Fig S1A. *, statistically significant from HER2 WT at >99% probability; †, statistically significant from mock treated at >99% probability; ns, not significant as compared with mock treated.
**Figure 2.** HER2 mutations cause resistance to EGFR antibodies. A and B, DiFi cell lines were treated with cetuximab (A) or panitumumab (B) for 5 days, and cell growth was measured by Alamar blue. C and D, NCI-H508 cell lines were treated with cetuximab (C) or panitumumab (D) for 5 days, and cell growth was measured by crystal violet assay. E, DiFi parental, HER2 WT, or HER2 V842I cells were treated with cetuximab for 24 hours and then lysed. Cell lysates were analyzed by Western blot. F, identical experiment performed on NCI-H508 cells.

Mutant HER2 was inhibited by neratinib and afatinib, with IC_{50} values ranging from 0.2 to 3 nmol/L (Fig. 3C and D). Parental DiFi and NCI-H508 cells are EGFR-dependent cell lines, and their growth is inhibited by neratinib or afatinib because these drugs inhibit both EGFR and HER2.

The effect of neratinib and afatinib on cell signaling was tested. Both drugs strongly inhibited HER2, EGFR, AKT, and MAPK phosphorylation in DiFi and NCI-H508 cells (Fig. 3E and F). The effect of neratinib or cetuximab on NCI-H508 cell growth was also tested in vivo using cell line xenografts.
**Figure 3.** HER2 mutants are highly sensitive to second-generation, irreversible HER2/EGFR tyrosine kinase inhibitors. **A and B,** DiFi cell lines were treated with neratinib (A) or afatinib (B) for 5 days, and cell growth was measured by Alamar blue. **C and D,** NCI-H508 cell lines were treated with neratinib (C) or afatinib (D) for 5 days, and cell growth was measured by crystal violet assay. **E,** DiFi parental, HER2 WT, or HER2 V842I cells were treated with neratinib (0.5 μmol/L) or afatinib (0.5 μmol/L) for 4 hours, and total lysates (8–10 μg) were harvested and analyzed by Western blot. **F,** identical experiment performed on NCI-H508 cells.
HER2 Activating Mutations Are Targets in Colorectal Cancer

(Neutrinib or the combination of neratinib plus trastuzumab inhibited the growth of NCI-H508 cells transduced with HER2<sup>V777L</sup> mutant colorectal cancer cell lines were transduced with HER2<sup>V777L</sup> and HER2<sup>WT</sup> cells grew in the presence of cetuximab (Supplementary Fig. S2B and S2C).

Comparison of the effect of neratinib between KRAS<sup>WT</sup> and mutant colorectal cancer cell lines was made (Supplementary Fig. S3). DiFi cells are KRAS, NRAS, BRAF, and PIK3CA WT (22). NCI-H508 cells are KRAS<sup>WT</sup> and NRAS<sup>WT</sup>, have an inactivating BRAF mutation (G596R), and have a PIK3CA<sup>E545K</sup> mutation, which is a well-known EGFR-activating mutation (23, 24). SW480 and HCT116 colorectal cancer cell lines have KRAS G12V and G13D mutations, respectively (23). These KRAS-mutated cell lines are relatively resistant to neratinib (IC<sub>50</sub> values of 430 nmol/L) compared with the KRAS<sup>WT</sup> cell lines, paralleling the results obtained with IMCE–KRAS cells (Supplementary Figs. S1B and S3). These results show that HER2-mutated cell lines, but not KRAS-mutated cell lines, are sensitive to the tyrosine kinase inhibitors neratinib and afatinib.

Colorectal PDXs with HER2 Mutations

Multiple mechanisms of resistance to EGFR antibodies have been reported, such as mutations in KRAS, NRAS, BRAF, and PIK3CA, or gene amplifications in HER2 and MET (4, 25). Cetuximab response rate in patients lacking these genetic alterations is approximately 20% to 25%, suggesting that there are additional factors contributing to drug resistance (4). We sequenced the HER2 gene in 48 colorectal cancer PDX samples that are cetuximab resistant and are WT for KRAS, NRAS, BRAF, and PIK3CA (quadruple WT). Four of these PDXs had HER2 mutations, and the allele frequency of the HER2 mutation in the primary tumor (prior to implantation) and in the xenograft grown in the mice was measured by next-generation DNA sequencing (Fig. 4A). The HER2<sup>S310F</sup> mutation, found in PDX M122, was previously shown to be an activating mutation (7) and functions the same as the S310F mutation studied in IMCE cells (Fig. 1C and D). The allele frequency of this mutation increased in the PDX, likely due to enrichment of malignant cells in the xenograft relative to the primary tumor. PDX M051 had both HER2 amplification and a novel kinase domain mutation, L866M. The allele frequency of L866M (0.968 to 0.986) indicated that the mutation is located on the amplified copies of the HER2 gene. HER2<sup>L866M</sup> is homologous to the EGFR<sup>L858R</sup> mutation, which is a well-known EGFR-activating mutation found in NSCLC (Fig. 4B; ref. 15). An in vitro kinase assay demonstrated that HER2<sup>L866M</sup> produced a 3-fold increase in tyrosine kinase activity relative to HER2<sup>WT</sup> (Fig. 4B). Both PDX M102 and M107 contained HER2<sup>V777L</sup> kinase domain mutations, and the allele frequency of 0.315 to 0.324 in M107 may represent a subclonal mutation. Cetuximab treatment of these four PDXs was previously performed (4) and demonstrated that these PDXs have de novo resistance to cetuximab (Supplementary Fig. S4A–S4D).

We tested the effect of HER2-targeted drugs on PDX M122 and M051. PDX M102 and M107 had previously been cryopreserved and could not be recovered during the timeframe of this project. For PDX M122 (Fig. 4C), treatment with trastuzumab, neratinib, or lapatinib alone delayed tumor growth, but after 30 days, the mice developed large tumors and had to be sacrificed. In contrast, dual HER2-targeted therapy with either trastuzumab plus neratinib or trastuzumab plus lapatinib produced tumor regression and absence of tumor regrowth during the 41-day window of this experiment. For PDX M051, which has HER2<sup>L866M</sup> kinase domain mutation plus HER2 gene amplification (Fig. 4D), treatment with trastuzumab had minimal effect on tumor growth. Neratinib as a single agent resulted in stable tumor size, whereas the combination of trastuzumab plus neratinib caused tumor regression, which was sustained over the duration of the experiment. After the final time point in both PDX experiments, the mice were sacrificed and the tumors excised. The tumor histology with both PDXs demonstrates that dual HER2-targeted therapy caused reduction in tumor cellularity and acquisition of more differentiated features (Supplementary Figs. S5 and S6). IHC on PDX M122 showed that treatment with neratinib or lapatinib (alone and, to a greater extent, when combined with trastuzumab) strongly reduced Ki-67, phosphoMAPK, and phosphoS6 immunoreactivity (Supplementary Figs. S5 and S6) but did not induce detectable signs of apoptosis (not shown). Trastuzumab alone was poorly effective at decreasing Ki-67, phosphoMAPK, and phosphoS6 levels. This lack of pharmacodynamic activity of trastuzumab alone was particularly evident in the IHC results from PDX M051, consistent with less therapeutic efficacy in vivo (Supplementary Figs. S5 and S6).

In order to understand this difference in trastuzumab activity between these two PDXs, we confirmed that HER2<sup>L866M</sup> activated intracellular signaling pathways in IMCE and NCI-H508 cells, producing resistance to EGFR monoclonal antibodies, and was sensitive to neratinib or afatinib in DiFi and NCI-H508 cells, similar to the other HER2 mutations tested in this study (Supplementary Fig. S7, Figs. 2 and 3). Comparison of trastuzumab sensitivity of HER2<sup>L866M</sup> versus HER2<sup>S310F</sup> mutation transduced cells suggests that the S310F mutation may have greater sensitivity to trastuzumab. NCI-H508 cells with HER2<sup>S310F</sup> were more sensitive to trastuzumab than HER2<sup>WT</sup> transduced cells, whereas resistance to trastuzumab was produced in cells transduced with HER2<sup>L866M</sup> (Supplementary Fig. S8). Similarly, in soft-agar assays on IMCE cells, trastuzumab had a greater effect on S310F-containing cells than L866M cells (Fig. 1D). Trastuzumab has multiple mechanisms of action on HER2-expressing cells (26). Detailed studies on these mechanisms are beyond the scope of this study and will be examined in the future.

To assess the specificity of these HER2-targeted drugs in this colorectal cancer PDX model system, we tested the effects of cetuximab, neratinib, and trastuzumab plus neratinib on a KRAS-mutant PDX (Fig. 4E). Unlike HER2-mutant PDXs, the KRAS-mutant PDX (PDX M551) continued to grow when treated with neratinib or trastuzumab plus neratinib. KRAS mutation is a known mechanism of resistance to cetuximab, and compared with the cetuximab treatment arm, the neratinib or trastuzumab plus neratinib arms had similar or slightly greater tumor growth. In total, these PDX experiments suggest that dual HER2-targeted therapy with trastuzumab plus neratinib may be an effective treatment for HER2-mutated, but not KRAS-mutated, colorectal cancers.
DISCUSSION

The TCGA project has identified HER2 somatic mutations from colorectal cancer patients, but the clinical effect of these mutations was unknown. Here, we show that these HER2 mutations activate intracellular signaling pathways, increase anchorage-independent growth in soft agar, and produce resistance to the EGFR monoclonal antibodies cetuximab and panitumumab in colon cell lines. HER2-mutant transduced DiFi and NCI-H508 cells are inhibited by low nanomolar doses of the second-generation, irreversible tyrosine kinase inhibitors neratinib and afatinib. Further, HER2 gene sequencing on 48 cetuximab-resistant, quadruple WT colorectal cancer PDXs identified 4 PDXs with HER2 activating mutations.
mutations (4/48 = 8.3%). The effect of HER2-targeted therapies on two of these PDXs was tested. Single-agent HER2-targeted therapy, with either trastuzumab, neratinib, or lapatinib, delayed the growth of these PDXs, and dual HER2-targeted therapy with either trastuzumab plus neratinib or trastuzumab plus lapatinib produced durable tumor regression in the mice. These results are consistent with recent analyses of the genomic landscape of response to EGFR therapy that identified sequence alterations in HER2 as a mechanism of resistance to cetuximab in colorectal cancer (Bertotti, Papp, and colleagues, Nature, in press). More importantly, these data suggest that HER2 activating mutations may themselves be a drug target for the treatment of colorectal cancer. These preclinical findings should be tested in colorectal cancer clinical trials.

Potential caveats and limitations of this study include the following. First, we acknowledge that retroviral transduction of HER2 into cell lines can produce overexpression of HER2. However, the colorectal cancer PDX samples contain endogenous HER2 mutations that are expressed at their native levels. The growth suppression of the PDXs by HER2-targeted agents demonstrates that HER2 activating mutations are required for the growth of these cancers. Second, next-generation genome sequencing has identified large numbers of HER2 somatic mutations, and it is not practical to experimentally test every mutation. Insights from structural biology and homology to known activating mutations in related genes as well as mutation impact prediction algorithms can generate hypotheses about the effect of novel mutations (6, 27, 28). Five mutations that were seen in only one patient with colorectal cancer (I263T, A466T, R678Q, R866W, and N1219S) were not tested in this study and should currently be regarded as variants of unknown significance.

There are many important implications of this study. HER2 somatic mutations are found in a wide range of solid tumors, including 9% of bladder cancer cases, 7% of glioblastoma cases, 5% of gastric cancer cases, 4% of lung adenocarcinoma cases, 3% of esophageal cancer cases, and 1.5% to 2% of breast cancer cases (16, 29). The broad distribution of HER2 somatic mutations suggests that HER2 activating mutations are drivers in a wide range of cancer types. The data presented here on colorectal cancer combined with prior functional studies on HER2 mutations in breast cancer and NSCLC support this hypothesis (6, 7). Further, a multi-institutional, phase II clinical trial is currently evaluating neratinib therapy in patients with a broad spectrum of solid tumors that harbor HER2 mutations (30).

These data form a strong preclinical rationale for clinical trials targeting HER2 activating mutations in patients with metastatic colorectal cancer. While this article was under review, Kloth and colleagues published a study indicating that 15% of Lynch syndrome or Lynch-like colorectal cancers have HER2 mutations, and they showed that the HER2-mutant colorectal cancer cell lines CW-2 and CCK-8 are sensitive to treatment with neratinib and afatinib (31). Our PDX results suggest that dual HER2-targeted therapy may be needed to achieve optimum antitumor effect. Several large clinical trials on HER2-amplified breast cancer have demonstrated improved patient outcomes with dual HER2-targeted therapy (32, 33). Metastatic colorectal cancers are routinely tested for KRAS, NRAS, and BRAF mutations (34). Our findings suggest that HER2 gene sequencing should also be included in this testing. With the growing availability of gene panels, testing metastatic colorectal cancer for multiple genes is now practicable. KRAS-mutated samples show resistance to neratinib in our experiments, and it would be prudent for current clinical trials to focus on colorectal cancer patients whose tumor is KRAS\textsuperscript{WT}. NSABP Oncology Genome Assessment Guided Mediation (N-GAMe) Program and the NSABP Colorectal Cancer Biospecimen Profiling Repository Trial (MPR-1 trial) include testing for HER2 mutations, and this will lead to prospective clinical trials for colorectal cancer patients.

**METHODS**

**Antibodies and Inhibitors**

Antibodies for Western blots were purchased from Cell Signaling Technologies: phosphoPLC\textgamma(Tyr783), PLC\textgamma, phosphoEGFR (Tyr1173), EGFR, phosphop44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phosphoAKT (Ser473), and AKT; Millipore: phosphoHER2 (p\textgamma1248); and Thermo Fisher: HER2 antibody (Ab-17). Antibodies for IHC were purchased from Cell Signaling Technology (phosphoS6 Ser235/236, clone D572.2E; phosphoERK1/2 Thr202/Tyr204, clone D13.144E) or Dako (Ki-67, clone MIB-1). Cetuximab, panitumumab, trastuzumab, and lapatinib were obtained from the hospital pharmacy. Afatinib was obtained from Selleckchem. Neratinib was provided by Puma Biotechnology, Inc., under a Materials Transfer Agreement.

**Cell Lines**

IMCE and IMCE-KRAS cells were a generous gift from Dr. Robert Whitehead (Vanderbilt University, Nashville). Dates of receipt were August 13, 2013, and November 7, 2014, respectively, for these two cell lines, and they were cultured in RPMI-1640 supplemented with 5% FCS, 1 µg/mL insulin, 10 µmol/L a-glucagon, 1 µmol/L hydrocorti- sone, 5 units per mL of mouse gamma interferon, and 1% penicillin/streptomycin (P/S) in a 5% CO\textsubscript{2} humidified atmosphere at 33°C. Cell line authentication of the IMCE cells (performed by Promega/ATCC on January 15, 2015) confirmed that they were a nonhuman cell line. DiFi cells were a gift from Dr. Alberto Bardelli (University of Torino, Italy; date of receipt May 13, 2013), and short tandem repeat profiling performed by Promega/ATCC on January 15, 2015, showed that they had a D5S818 11,12; D1S317 8,11; D7S820 10,12; D16S539 12; vWA 17,18; THO1 7, 9.3; AMEL X; TPOX 8,9; and CSF1PO 10,11 profile. NCI-H508 cells were purchased from the ATCC (date of receipt May 29, 2014) and not further authenticated. The ATCC performs authentication on its own cell lines, and the NCI-H508 cells were used for fewer than 6 months after receipt and resuscitation from cryopreservation. SW480 and HCT116 cell lines were obtained from Drs. Jeya Shao and David Pwnica-Worms (Washington University School of Medicine, St. Louis, MO; date of receipt May 14, 2014), and no authentication was performed on these cells. DiFi and NCI-H508 cells were maintained in a 5% CO\textsubscript{2} humidified atmosphere at 37°C, and culture media for these two cell lines are as follows: DiFi cells: F12 medium supplemented with 5% FCS, 1% P/S; NCI-H508 cells: RPMI-1640 supplemented with 5% FCS, 1% P/S. Inhibition of cell growth by cetuximab, panitumumab, trastuzumab, and lapatinib was measured by Alamar blue or crystal violet assay (35). IC\textsubscript{50} values were calculated by a 4-parameter nonlinear regression conducted using SigmaPlot version 11 software (Systat software, Inc).

**Retroviral Transduction of HER2 Mutants in Colorectal Cancer Cell Lines**

HER2 WT or mutant retroviral vectors that we published were transfected in ONX amphotropic packaging cell line. HER2 WT or mutant recombinant retroviral supernatants were transduced in IMCE, DiFi, and NCI-H508 cell lines as described previously (6). After 2 to 3 weeks of zeocin selection of bulk infected cultures, transgene expression was verified by FACS analysis for GFP expression.
Soft-Agar Colony-Forming Assay

Six-well plates were first layered with 0.6% bacto agar in IMCE growth medium. After the solidification of the bottom layer, a top layer containing 5 × 10^3 to 10 × 10^3 IMCE HER2 WT or mutant cells in IMCE growth medium plus 0.4% bacto agar was added. Assays were carried out in duplicate. Cells were allowed to form colonies for 12 days and were photographed and quantitated as shown previously (6).

Statistical analysis of the colony count data was modeled using Poisson regression using the MCMCglmm package (version 2.16; ref. 36), of the R statistical environment (version 2.15.1) using “Genotype” and a “Genotype:Treatment” interaction as fixed predictors. No intercept was included to force explicit measurements for each “Genotype.” MCMCglmm uses fully Bayesian modeling and parameter estimates generated using Gibbs sampling Markov Chain Monte Carlo with 100,000 iterations, a burn-in of 3,000, and a thin of 10. Diagnostics revealed the lack of autocorrelation and excellent chain mixing. The default prior was used for fixed effects, which is a multivariate normal distribution with a 0 mean vector and diagonal variance matrix with variances of 10^4 and covariances of 0, which ensures that fixed effects are independent and estimated almost entirely from the data. Overdispersion and replicates were accounted for in the residual variance structure with an improper inverse Wishart prior with nu = 0 and V = 1, which implicitly assumes each well is a random effect. Parameters and parameter contrasts were considered to be statistically significant when the 95% highest posterior density interval did not contain 0. The conclusions were robust to changes in the minimal colony size from 1 to 10 (the global median colony size).

HER2 Gene Sequencing and In Vitro Kinase Assay

Initial screening of PDX samples for HER2 mutations was conducted by Sanger sequencing. Genomic DNA was extracted with the Wizard Purification System (Promega). Primers for HER2 exons 8 and 18–24 were designed with Primer3 software and synthesized by Sigma. Purified PCR products were sequenced with a BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed with a 3730 ABI capillary electrophoresis system. Confirmation of HER2 mutations and determination of allele frequency were performed by next-generation sequencing using an Illumina MiSeq instrument. Briefly, all ERBB2 exons were PCR amplified in triplicate (75 ng DNA per reaction) on a BioMark HD system (Fluidigm). All samples were pooled and cleaned using bead purification. The samples were loaded on an Illumina MiSeq instrument and sequenced. Total read counts at the nucleotide position of the identified HER2 mutations ranged from 13,600 to 65,100 reads. The HER2 tyrosine kinase domain was recombinantly expressed in Sf9 cells using a baculoviral vector and purified to greater than 80% purity, as previously described (6, 37). In vitro kinase assays were performed using γ-32P-ATP and a synthetic peptide substrate (37).

 Xenograft Models and In Vivo Treatments

Tumor implantation and expansion were performed as previously described (4). Established tumors (average volume, 400–600 mm³) were treated with the following regimens, either single-agent or in combination: trastuzumab (Roche) 30 mg/kg weekly (vehicle: physiologic saline); neratinib (Puma Biotechnology) 40 mg/kg orally daily (vehicle, 0.5% methylcellulose, 0.4% Tween-80). Tumor size was evaluated once weekly by caliper measurements, and the volume of the mass was calculated using the formula 4/3×π×(d/2)²×(D/2), where d is the minor tumor axis and D is the major tumor axis. All values for tumor growth curves were recorded blindly. In vivo procedures and related biobanking data were managed using the Laboratory Assistant Suite (LAS), a web-based proprietary data management system for automated data tracking (38). Animal procedures were approved by the Ethical Commission of the Candiolo Cancer Institute and by the Italian Ministry of Health.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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REFERENCES


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