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HER2 activating mutations are targets for colorectal cancer treatment

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Abstract

The Cancer Genome Atlas project identified HER2 somatic mutations and gene amplification in 7% of colorectal cancer patients. 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 mutations 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) WT colorectal cancer patient-derived xenografts (PDX's) identified 4 PDX's with HER2 mutations. HER2 targeted therapies were tested on two PDX's. 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 PDX's.

Keywords

HER2; cetuximab; targeted therapy; colorectal cancer; protein tyrosine kinases; kinase inhibitors

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Introduction

Cancer genome sequencing is identifying new genetic alterations and new driver events in human cancers (1, 2). The Cancer Genome Atlas (TCGA) colorectal cancer project found that 7% of colorectal cancer (CRC) patients have HER2 somatic mutations or HER2 gene amplification (3). HER2 gene amplification in CRC is known to produce resistance to the EGFR monoclonal antibodies, cetuximab and panitumumab (4, 5). To our knowledge, the impact of HER2 somatic mutations in CRC has not been studied and it is open question as to whether HER2 mutations are clinically important in CRC.

HER2 somatic mutations have been previously studied in breast and non-small cell lung cancers (NSCLC). The majority of these mutations were shown to be activating mutations 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 pre-clinical data have led to two multi-institutional, phase II clinical trials that will screen metastatic breast cancer patients 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 are demonstrating clinical efficacy of combining neratinib with the mTOR inhibitor, temsirolimus (11, 12).

In this study, we determined the effect of HER2 somatic mutations in CRC. The HER2 mutations found in CRC 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 CRC patient-derived xenografts (PDX) and demonstrate that dual HER2 targeted therapy causes tumor regression. These data form a strong pre-clinical rationale for clinical trials targeting HER2 activating mutations in metastatic CRC patients.

Results

HER2 mutations identified from colorectal cancer patients cause oncogenic transformation of colon epithelial cells

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 has been described for other oncogenes, including Ras and EGFR (13). In addition to TCGA colorectal cancer study, a recent sequencing study on CRC patients 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 breast cancer patients, 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 CRC sequencing studies (8, 15). The HER2 kinase mutations V777L and V842I were seen in multiple patients, with 2 cases of HER2

V777L and 4 cases of HER2 V842I identified (Fig. 1B). Half (6/12) of these HER2 mutated CRC case are KRAS WT and one-third (4/12) of these case 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 (KRAS K117N and A146T). The co-occurring BRAF mutations in HER2 mutated cases included one case with BRAF V600E (TCGA-AA-3947) and one case with BRAF F247L (TCGA-AG-A002). The BRAF F247L mutation is located in the C1 domain of BRAF, it has not been reported in any other sample in the cBioPortal and 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 side 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) are 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 cells (IMCE) and measured their effect on cell signaling and anchorage independent growth. IMCE cells are non-transformed 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, MAP kinase (MAPK), and Akt phosphorylation seen relative to the HER2 WT transduced cells (Fig. 1C). Phosphorylation of the immediate HER2 substrate, phospholipase γ C1 (PLC γ) was greatest in the HER2 V777L 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 and Supp. Fig. S1A). Interestingly, in our prior study which used MCF10A immortalized breast epithelial cell line, the HER2 L755S mutation did not increase soft agar colony formation or alter colony morphology in Matrigel (6). This difference in soft agar colony formation between MCF10A-HER2 L755S and IMCE-HER2 L755S 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 50–100nM neratinib, whereas IMCE-HER2 V842I cells show an IC₅₀ of approximately 10nM neratinib in the soft agar colony formation assay (Supp. 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 HER2 V842I mutation into the cetuximab sensitive colorectal cell line, DIFI, using a retroviral vector (Fig. 2A–B). We initially focused on the HER2 V842I mutation because it was the most prevalent mutation

identified in CRC cases by TCGA (3). Introduction of WT HER2 into DIFI cells caused a modest (2–4 fold) change in the cell's sensitivity to cetuximab and panitumumab (red curves, Fig. 2A–B). In contrast, introduction of the V842I mutation caused a 40–100 fold shift in the IC₅₀ values (blue curves, Fig. 2A–B). NCI-H508 cells are 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–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–F). Introduction of HER2 mutations into these cells increased MAPK and EGFR phosphorylation and this was sustained even in the presence of cetuximab.

HER2 mutations 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, and DIFI-HER2 V842I cells with IC₅₀ values ranging from 2–5 nM (Fig. 3A–B). Similarly, parental NCI-H508 cells and cells transduced with WT or mutant HER2 were growth inhibited by neratinib and afatinib with IC₅₀ values ranging from 0.2–3 nM (Fig 3C–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–F). The effect of neratinib or cetuximab on NCI-H508 cell growth was also tested *in vivo* using cell line xenografts (Supp. Fig. S2). Neratinib or the combination of neratinib +trastuzumab inhibited the growth of NCI-H508 cells transduced with HER2 V777L. Cetuximab inhibited the growth of NCI-H508 parental cells (Supp. Fig. S2A), whereas NCI-H508 HER2 V777L and HER2 WT cells grew in the presence of cetuximab (Supp. Fig. S2B–C).

Comparison of the effect of neratinib between KRAS WT and mutant colorectal cancer cell lines was made (Supp. Fig. S3). DIFI cells are KRAS, NRAS, BRAF, and PIK3CA WT (22). NCI-H508 cells are KRAS WT, NRAS WT, have an inactivating BRAF mutation (G596R) and have a PIK3CA E545K helical domain mutation (23, 24). SW480 and HCT116 colorectal cancer cells have KRAS G12V and G13D mutations, respectively (23). These KRAS mutated cell lines are relatively resistant to neratinib (IC₅₀ values of 430 nM) compared to the KRAS WT cell lines, paralleling the results obtained with IMCE-KRAS cells (Supp. Fig. S3 and S1B). 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 patient derived xenografts 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 20–25%, suggesting that there are additional factors contributing to drug resistance (4). We sequenced the HER2 gene in 48 CRC PDX samples that are cetuximab resistant and are WT for KRAS, NRAS, BRAF, PIK3CA (quadruple WT). Four of these PDX's 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 S310Y 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–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) indicates that the mutation is located on the amplified copies of the HER2 gene. HER2 L866M is homologous to the EGFR L858R mutation, which is a well-known EGFR activating mutation found in NSCLC (Fig. 4B) (15). An *in vitro* kinase assay demonstrated that HER2 L866M produced a 3-fold increase in tyrosine kinase activity relative to WT HER2 (Fig. 4B). PDX's M102 and M107 both contained HER2 V777L 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 PDX's was previously performed (4) and demonstrated that these PDX's have *de novo* resistance to cetuximab (Supp. Fig. S4A–D).

We tested the effect of HER2 targeted drugs on PDX's M122 and M051. PDX's 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 on their own 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 +neratinib or trastuzumab+lapatinib produced tumor regression and absence of tumor re-growth during the 41 day window of this experiment. For PDX M051 which has HER2 L866M kinase domain mutation plus 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+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 PDX's demonstrates that dual HER2 targeted therapy caused reduction in tumor cellularity and acquisition of more differentiated features (Supp. Fig. S5 and S6). Immunohistochemistry (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 (Supp. Fig. 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* (Supp. Fig. S5 and S6).

In order to understand this difference in trastuzumab effect between these two PDX's, we confirmed that HER2 L866M activated intracellular signaling pathways in IMCE and NCI-H508 cells, produced 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 (Supp. Fig. S7, and Figures 2 and 3). Comparison of trastuzumab sensitivity of HER2 L866M versus HER2 S310F mutation transduced cells suggests that the S310F mutation may have greater sensitivity to trastuzumab. NCI-H508 cells with HER2 S310F were more sensitive to trastuzumab than HER2 WT transduced cells, whereas resistance to trastuzumab was produced in cells transduced with HER2 L866M (Supp. 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 CRC PDX model system, we tested the effects of cetuximab, neratinib, and trastuzumab+neratinib on a KRAS mutant PDX (Fig. 4E). Unlike the effects on HER2 mutant PDX's, the KRAS mutant PDX (PDX M551) continued to grow when treated with neratinib or trastuzumab+neratinib. KRAS is a known mechanism of resistance to cetuximab and compared to the cetuximab treatment arm, the neratinib or trastuzumab+neratinib arms had similar or slightly greater tumor growth. In total, these PDX experiments suggest that dual HER2 targeted therapy with trastuzumab +neratinib may be an effective treatment for HER2 mutated, but not KRAS mutated, colorectal cancers.

Discussion

The Cancer Genome Atlas (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 CRC PDX's identified 4 PDX's with HER2 activating mutations ($4/48 = 8.3\%$). The effect of HER2 targeted therapies on two of these PDX's was tested. Single agent HER2 targeted therapy, with either trastuzumab, neratinib, or lapatinib, delayed the growth of these PDX's and dual HER2 targeted therapy with either trastuzumab+neratinib or trastuzumab+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 et al, 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 pre-clinical 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 CRC PDX samples contain endogenous HER2 mutations which are expressed at their native levels. The growth suppression of the PDX's 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 CRC patient (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, 3% of esophageal cancer, and 1.5–2% 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 CRC combined with prior functional studies on HER2 mutations in breast cancer and NSCLC supports 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 harboring HER2 mutations (30).

These data form a strong pre-clinical rationale for clinical trials targeting HER2 activating mutations in metastatic CRC patients. While this paper was under review, Kloth and colleagues published that 15% of Lynch syndrome or Lynch-like CRC have HER2 mutations and they showed that the HER2 mutant CRC cell lines, CW-2 and CCK-81, are sensitive to treatment with neratinib and afatinib (31). Our PDX results suggest that dual HER2 targeted therapy may be needed to achieve optimum anti-tumor effect. Several large, clinical trials on HER2 amplified breast cancer have demonstrated improved patient outcomes with dual HER2 targeted therapy (32, 33). Metastatic CRC's 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 CRC for multiple genes is now practical. KRAS mutated samples show resistance to neratinib in our experiments and it would be prudent for current clinical trials to focus on CRC patients whose tumor is KRAS WT. The NSABP Oncology Genome Assessment Guided Medicine (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 CRC patients.

Methods

Antibodies and Inhibitors

Antibodies for western blots were purchased from Cell Signaling Technologies: phospho PLC γ (Tyr783), PLC γ , phospho EGFR (Tyr1173), EGFR, phospho p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho Akt (Ser473), and Akt; Millipore: phospho HER2

(pY1248); and Thermo Fisher: HER2 antibody (Ab-17). Antibodies for immunohistochemistry were purchased from Cell Signaling Technology (phospho-S6 Ser235/236, clone D57.2.2E; phospho-ERK1/2 Thr202/Tyr204, clone D13.14.4E) or Dako (Ki-67, clone MIB-1). Cetuximab, panitumumab, trastuzumab, and lapatinib were obtained from the hospital pharmacy. Afatinib was obtained from Selleckchem (Houston, TX). 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, USA). Date of receipt was Aug. 13, 2013 and Nov. 7, 2014, respectively for these two cell lines and they were cultured in RPMI-1640 supplemented with 5% fetal calf serum, 1µg/ml Insulin, 10µM α-thioglycerol, 1µM hydrocortisone, 5 units per ml of mouse gamma interferon and 1% penicillin/streptomycin (P/S) in a 5% CO₂ humidified atmosphere at 33°C. Cell line authentication of the IMCE cells confirmed that they are a non-human cell line (performed by Promega/ATCC on Jan. 15, 2015). DIFI cells were a gift from Dr. Alberto Bardelli (University of Torino, Italy – date of receipt May 15, 2013) and STR profiling performed by Promega/ATCC on January 15, 2015 showed that it had a D5S818 11,12; D13S317 8,11; D7S820 10,12; D16S539 12; vWA 17,18; TH01 7, 9.3; AMEL X; TPOX 8,9; and CSF1PO 10,11 profile. NCI-H508 cells were purchased from the American Type Culture Collection (ATCC, date of receipt 5/29/14) and not further authenticated. 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 cells were obtained from Drs. Jieya Shao and David Piwnica-Worms (date of receipt May 2014) and no authentication was performed on these cells. DIFI and NCI-H508 cells were maintained in a 5% CO₂ humidified atmosphere at 37 °C and culture media for these two cell lines is as follows: DIFI cells – F12 medium supplemental with 5% fetal calf serum, 1% P/S. NCI-H508 cells were grown in RPMI-1640 supplemented with 5% fetal calf serum, 1% P/S. Inhibition of cell growth by cetuximab, panitumumab, neratinib and afatinib was measured by Alamar blue or crystal violet assay (35). IC₅₀ values 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 transfected in ØNX amphotropic packaging cell line. HER2 WT or mutants recombinant retroviral supernatants were transduced in IMCE, DIFI, and NCI-H508 cell lines as described previously (6). After 2–3 weeks of zeocin selection of bulk infected cultures, transgene expression is verified by FACS analysis for GFP expression (always >90%). Western blot analysis was performed on polyclonal cell lines to confirm HER2 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 bottom layer, a top layer containing 5–10×10³ IMCE HER2 WT or mutants were re-suspended in IMCE growth medium containing 0.4% bacto agar was added. Assays

were carried out in duplicates. Cells were allowed to form colonies for 12 days and photographed and quantified as shown previously (6).

Statistical analysis of the colony count data were modeled using Poisson regression using the MCMCglmm package (version 2.16) (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 3000, 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^{10} and covariances of 0, as this ensures 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). Exon-specific for HER2 exons 8 and 18–24 were designed with Primer3 software and synthesized by Sigma. Purified PCR products were sequenced with 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 was performed by NGS using an Illumina MiSeq instrument. Briefly, all ERBB2 exons were PCR amplified in triplicate (75ng DNA per reaction) on a BioMark HD system (Fluidigm, South San Francisco, CA). All samples were pooled and cleaned using bead purification. The samples were loaded on an Illumina MiSeq instrument (San Diego, CA) and sequenced. Total read counts at the nucleotide position of the identified HER2 mutations ranged from 13,600 to 65,100 reads. HER2 tyrosine kinase domain was recombinantly expressed in Sf9 cells using a baculoviral vector and purified to >80% purity, as previously described (6, 37). *In vitro* kinase assays were performed using γ - ^{32}P -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: physiological 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 $\frac{4}{3} \times \pi \times (d/2)^2 \times (D/2)$, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation List

CRC	colorectal cancer
IHC	immunohistochemistry
IMCE	immortalized mouse colon epithelial cells
MAPK	mitogen activated protein kinase
MSS	microsatellite stability
MSI	microsatellite instability
NSCLC	non-small cell lung cancers
PDX	patient-derived xenografts
PLCγ	phospholipase γ C1
TCGA	The Cancer Genome Atlas

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Statement of Significance

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

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 CRC project. **B**, HER2 somatic mutations observed in 12 CRC patients are shown. Red circles represent TCGA cases with HER2 gene amplification, blue circles represent TCGA cases that do not have HER2 gene amplification, and green circles represent cases from Brannon et al., 2014 (14) and gene amplification is not reported on these cases. One TCGA case had concurrent V842I + V777L mutations along with gene amplification. FU=Furin-like domains. TM=transmembrane region. **C**, IMCE cells were retrovirally transduced with HER2 WT or mutants. 8–10 μ g of total lysates were analyzed by western blot. **D**, IMCE HER2 WT or HER2 mutants were seeded in soft agar in duplicate, treated with trastuzumab (100 μ g/ml) or neratinib (500nM), allowed to grow for 12 days, and stained with crystal violet. Photographs of the stained wells are shown in Supp. Fig S1A.

*Statistically significant from HER2 WT at >99% probability. †Statistically significant from mock treated at >99% probability. ns=not significant as compared to mock treated.

Figure 2. HER2 mutations cause resistance to EGFR antibodies. **A, B**, DIFI cell lines were treated with cetuximab (A) or panitumumab (B) for 5 days and cell growth was measured by Alamar blue. **C, 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 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.

Figure 3.

HER2 mutations are highly sensitive to second generation, irreversible HER2/EGFR tyrosine kinase inhibitors. **A, B**, DIFI cell lines were treated with neratinib (**A**) or afatinib (**B**) for 5 days and cell growth was measured by Alamar blue. **C, 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 V842I cells were treated with neratinib (0.5μM) or afatinib (0.5μM) for 4hrs and 8–10ug of total lysates were harvested and analyzed by western blot. **F**, Identical experiment performed on NCI-H508 cells.

Figure 4. Drug treatment of HER2 or KRAS mutant CRC patient derived xenografts (PDX). **A**, HER2 gene specific sequencing of 48 cetuximab refractory, quadruple WT PDX's identified 4 PDX's with HER2 mutations. Allele frequencies from next-generation DNA sequencing on the primary tumor prior to implantation or of the PDX grown in the mice is shown. **B**, *In vitro* kinase assay on WT or L866M HER2 kinase domain. HER2 L866M is homologous to EGFR L858R. **C–E**, Tumor growth curves for PDX's tumors (n=5 for each treatment arm). Data represent mean $\pm$ S.E.M. Drug doses are: trastuzumab 30 mg/kg weekly, neratinib 40 mg/kg orally daily, lapatinib 100 mg/kg orally daily, and cetuximab 20mg/kg twice weekly.