Original Article

Targeting HER2 in Colorectal Cancer: The Landscape of Amplification and Short Variant Mutations in *ERBB2* and *ERBB3*

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BACKGROUND: In contrast to lung cancer, few precision treatments are available for colorectal cancer (CRC). One rapidly emerging treatment target in CRC is *ERBB2* (human epidermal growth factor receptor 2 [HER2]). Oncogenic alterations in HER2, or its dimerization partner HER3, can underlie sensitivity to HER2-targeted therapies. **METHODS:** In this study, 8887 CRC cases were evaluated by comprehensive genomic profiling for genomic alterations in 315 cancer-related genes, tumor mutational burden, and microsatellite instability. This cohort included both colonic (7599 cases; 85.5%) and rectal (1288 cases; 14.5%) adenocarcinomas. **RESULTS:** A total of 569 mCRCs were positive for *ERBB2* (429 cases; 4.8%) and/or *ERBB3* (148 cases; 1.7%) and featured *ERBB* amplification, short variant alterations, or a combination of the 2. High tumor mutational burden (≥20 mutations/Mb) was significantly more common in *ERBB*-mutated samples, and *ERBB3*-mutated CRCs were significantly more likely to have high microsatellite instability ($P<.002$). Alterations affecting *KRAS* (27.3%) were significantly underrepresented in *ERBB2*-amplified samples compared with wild-type CRC samples (51.8%), and *ERBB2*- or *ERBB3*-mutated samples (49.0% and 60.8%, respectively) ($P<.01$). Other significant differences in mutation frequency were observed for genes in the PI3K/MTOR and mismatch repair pathways. **CONCLUSIONS:** Although observed less often than in breast or upper gastrointestinal carcinomas, indications for which anti-HER2 therapies are approved, the percentage of CRC with *ERBB* genomic alterations is significant. Importantly, 32% of *ERBB2*-positive CRCs harbor short variant alterations that are undetectable by routine immunohistochemistry or fluorescence in situ hybridization testing. The success of anti-HER2 therapies in ongoing clinical trials is a promising development for patients with CRC. ***Cancer* 2018;124:1358-73.**

KEYWORDS: colorectal adenocarcinoma, comprehensive genomic profiling, *ERBB2, ERBB3*, human epidermal growth factor receptor 2 (HER2), lapatinib, microsatellite instability, pertuzumab, trastuzumab, tumor mutational burden.

INTRODUCTION

Altered human epidermal growth factor receptor 2 (HER2) signaling caused by genomic amplification of *ERBB2* or mutations is oncogenic and has been observed in multiple cancer types.[1-3] Amplification of wild-type (nonmutated) *ERBB2* is observed in 15% to 20% of breast carcinomas and a similar proportion of gastric and gastroesophageal junction adenocarcinomas.[4-6] This observation led to the development of therapeutic antibodies targeting this receptor, such as trastuzumab, pertuzumab, and ado-trasuzumab emtansine, as well as pan-ERBB small molecule inhibitors, such as lapatinib or afatinib.[7-11] To identify patients for whom anti-HER2 therapy is predicted to be most beneficial, fluorescence in situ hybridization (FISH) to evaluate *ERBB2* amplification and immunohistochemistry (IHC) to test for HER2 protein overexpression are routinely performed as part of the standard clinical care for breast and upper gastrointestinal tract adenocarcinomas.[4,5] In addition to copy number changes in *ERBB2*, genomic sequencing studies have identified missense mutations and small indels within the kinase domain of HER2 in approximately 2% of lung cancers.[12-14] Activating *ERBB2* mutations have also been found in approximately 2% of breast cancers and are enriched 10-fold in invasive lobular carcinomas that harbor concurrent *CDH1* mutations.[15-17] Similarly, activating extracellular domain mutations have been observed in approximately 40% of micropapillary urothelial carcinomas.[18] Extensive preclinical studies have demonstrated

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that these mutations are oncogenic and sensitive to inhibitors targeting HER2, and that targeting *ERBB2* mutations affecting either the kinase or extracellular domains has shown efficacy in a wide variety of tumor types.[11,19-25] Alterations in the HER2 dimerization partner HER3, encoded by *ERBB3*, can also activate HER2 signaling and underlie sensitivity to targeted therapies.[11,26-28]

Recent studies of *ERBB2* amplification and sequence mutations in colorectal cancer (CRC) suggest that HER2 is a therapy target in this disease,[29-33] in addition to being a mechanism of resistance to epidermal growth factor receptor (EGFR)-targeted therapies such as cetuximab and panitumumab.[34-38] Similarly, reports of high-level *ERBB3* amplification being a negative prognostic factor within the context of CRC suggest that HER3 may also be a target in this tumor type.[27,39,40] These studies encourage continued research into the effects and prevalence of *ERBB* alterations in patients with recurrent and metastatic CRC, as well as their importance for treatment. Data from the HERACLES[36] and MyPathway[41] studies demonstrate objective response rates of 30% to 38% for patients with HER2-overexpressing CRC who are treated with trastuzumab plus lapatinib or trastuzumab plus pertuzumab, respectively. In the following study of nearly 9000 clinically advanced and metastatic CRC (mCRC) cases, the relative frequencies of *ERBB* amplification and sequence alterations were evaluated and evidence of the clinical efficacy of anti-HER2 targeted therapies in *ERBB*-driven mCRC presented.

MATERIALS AND METHODS

Comprehensive genomic profiling was performed for 8887 consecutive cases of primarily recurrent CRC, refractory CRC, and mCRC during the course of routine clinical care. Approval for the current study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act (HIPAA) waiver of authorization, was obtained from the Western Institutional Review Board (protocol no. 20152817). The pathologic diagnosis of each case was confirmed on routine hematoxylin and eosin-stained slides and all samples forwarded for DNA extraction contained a minimum of 20% tumor nuclei.

The sequencing methods used for comprehensive genomic profiling, including validation of copy number and variant calling affecting *ERBB2*, have been described in detail elsewhere.[42] Sample processing and sequencing analysis was performed in a laboratory accredited under the Clinical Laboratory Improvement Amendments (CLIA) and by the College of American Pathologists (CAP). In brief, samples undergo pathologist review to ensure sufficient tumor material (minimum 20% tumor nuclei) and to resolve any conflicts with the provided histological description. From a minimum of 40 microns for each sample provided as formalin-fixed, paraffin-embedded tissue blocks, at least 50 ng of DNA was extracted. The samples were assayed using adaptor-ligation and hybrid capture next-generation sequencing (FoundationOne; Foundation Medicine, Cambridge, Massachusetts) for all coding exons from 287 (version 1) or 315 (version 2) cancer-related genes, plus select introns from 19 (version 1) or 28 (version 2) genes frequently rearranged in cancer (see Supporting Information Tables 1 and 2). Sequencing of captured libraries was performed using Illumina HiSeq technology (Illumina, San Diego, California) to a mean exon coverage depth of $>500\times$, and resultant sequences were analyzed using both an algorithmic pipeline and manual curation for base substitutions, small insertions or deletions, copy number alterations (amplifications and homozygous deletions), and select gene fusions, as previously described.[42] Clinically relevant genomic alterations were defined as alterations that are targetable by anticancer drugs currently available on the market or in registered clinical trials. Germline variants documented in the dbSNP database (dbSNP142; http://www.ncbi.nlm.nih.gov/SNP/), with ≥ 2 counts in the ExAC database (http://exac.broadinstitute.org/), or recurrent variants of unknown significance that were predicted by an internally developed algorithm to be germline were removed, with the exception of known driver events.[42] Confirmed somatic alterations deposited in the Catalog of Somatic Mutations in Cancer (COSMIC v62) were highlighted as biologically significant.[43] All inactivating events (ie, truncating mutations and deletions) in known tumor suppressor genes were also called as significant. To maximize mutation-detection accuracy (sensitivity and specificity) in impure clinical specimens, the test was optimized and validated to detect base substitutions at a $\geq$5% mutant allele frequency, indels with a $\geq$10% mutant allele frequency with $\geq$99% accuracy, and fusions occurring within baited introns/exons with $>$ 99% sensitivity.[42]

Each tumor sample is analyzed alongside an internally validated mixture of 10 heterozygous diploid HAPMAP control samples, which custom algorithms use to normalize the sequence coverage distribution across baited targets. Normalized coverage data for exonic, intronic, and single-nucleotide polymorphism (SNP) targets accounting for stromal admixture are plotted on a logarithmic scale and minor allele SNP frequencies are concordantly plotted across the genome. Further cluster groupings of targets and minor allele SNPs are used to define upper and lower bounds of genomic segments.

TABLE 1. Clinical and Genomic Characteristics of *ERBB2*- and *ERBB3*-mutated mCRC

	All mCRC	*ERBB2* Positive		*ERBB2* Positive	*ERBB3* Positive	Cooccurring *ERBB2/3*
		Amplification	Short Variants	Amp + SV		
No. of cases						
Total	8887	251 (2.8%)	135 (1.5%)	35 (0.4%)	140 (1.6%)	8 (0.1%)
Colonic CRC	7599	215 (2.8%)	112 (1.5%)	28 (0.4%)	113 (1.5%)	7 (0.1%)
Rectal CRC	1288	36 (2.8%)	23 (1.8%)	7 (0.5%)	27 (2.1%)	1 (<0.1%)
Sample site						
Colorectal	4660	124	79	21	64	4
Distant	4176	124	55	14	74	4
Stage						
IV	100%	100%	100%	100%	100%	100%
Patient demographics						
Median age (range), y	56 (8-96)	54 (22-88)	59 (31-79)	57 (29-87)	54 (14-83)	53 (46-80)
Sex						
Female	45%	43%	41%	46%	39%	50%
Male	55%	57%	59%	54%	60%	50%
ERBB mutation type						
Amplification	NA	251	-	-	2	0
Short variant	NA	-	135	-	138	8
Amp + SV	NA	-	-	35	0	0
Global mutation metrics						
TMB (mut/Mb)						
Range	0-854.1	0-230.6	0-230.6	0-10.1	0-854.1	6.3-126.1
Median	3.8	3.6	5.4	3.8	5.4	44.2
<6 mut/Mb	6294 (70.8%)	179 (71.3%)	68 (50.4%)	27 (77.1%)	79 (56.4%)	0 (0.0%)
6-20 mut/Mb	2173 (24.5%)	72 (28.7%)	38 (28.1%)	8 (22.9%)	36 (25.7%)	2 (25.0%)
≥20 mut/Mb	420 (4.7%)	0 (0%)	29 (21.5%)	0 (0%)	25 (17.9%)	6 (75.0%)
P	-	<<.0005	<.0001	NS	<<.0001	<<.0001
MSI						
No. of cases evaluated	5899	171	77	24	83	5
Stable	5389 (91.4%)	169 (98.8%)	64 (83.1%)	24 (100%)	69 (83.1%)	1 (20%)
Ambiguous	103 (1.7%)	2 (1.2%)	1 (1.3%)	0 (0%)	1 (1.2%)	1 (20%)
High	407 (6.9%)	0 (0%)	12 (15.6%)	0 (0%)	12 (14.5%)	3 (60%)
P	-	<.005	<.005	NS	<.05	<<.0001

Abbreviations: Amp, amplification; mCRC, metastatic colorectal cancer; MSI, microsatellite instability; NA, not applicable, NS, not significant; mut/Mb, mutations per megabase; TMB, tumor mutational burden; SV, short variant.
Samples in the cooccurring column had both *ERBB2* and *ERBB3* alterations, whereas other samples had only *ERBB2* or *ERBB3* alterations. Sample site was defined as colorectal for the colon or rectum and distant for all others; a subset of samples did not have the exact sample site defined. Significance values for TMB and MSI were calculated by the chi-square test and compared the distribution of samples positive for a given alteration type with the distribution for all other samples in the data set.

Empirical Bayesian algorithms use a distribution of parameters including purity and base ploidy and probability matrices are derived using different statistical sampling methodologies to fit these data and generate copy number alteration variant calls; all computational models are reviewed by expert analysts for each sample. Given that each copy number model is dynamically generated for each individual sample, credibility and confidence intervals vary with sample data; however, copy number calling achieves high performance (sensitivity was 99% with positive predictive value >99%) within a range of 20% to 75% tumor content, as previously described.[42]

Previous studies have demonstrated high levels of concordance between the current method of detecting *ERBB2* amplification and FISH.[42] In that study, 2 cohorts of breast carcinomas were shown to have 100% (42 of 42 cases) and 97% (29 of 30 cases) concordance with FISH results.[42] Amplification of *ERBB2* as described here includes the detection of ≥5 copies of *ERBB2* above the overall ploidy of the tumor sample.

Alteration nomenclature in general follows the recommendations of the Human Genome Variation Society.[44] Briefly, frameshift alterations are described as follows: first amino acid changed, position of first amino acid change, fs to designate frame shift variant and * to designate termination codon, position of termination site relative to first amino acid changed. A plus sign is used to designate that no termination site is encountered in that frame before the end of the normally encoded protein sequence. For example, A1232fs*25 + indicates the initiation of a frame shift event at alanine 1232, with 25 amino acids of novel sequence before the normal

TABLE 2. Short Variant Alterations Observed in *ERBB2*-Mutated and *ERBB3*-Mutated mCRC

ERBB2

Mutation Type	Alteration	Count	Cases With Multiple Alterations	
			SV Only	Amplification
Missense		N=181		
ECD				
	P122L	1		
	E265K	1		
	G292R	1		
	S310F	19		6
	S310Y	8	1	1
	L313V	1		
TM				
	V659E	1		1
	G660D	2		1
	S653C	1		1
JM				
	R678Q	44	1	3
KD				
	T733I	1		1
	L755S	12	3	1
	I767M	2		
	D769H	1		
	D769N	2		
	D769Y	6	3	1
	V773M	2		
	G776S	1		1
	G776V	6		
	V777L	19	2	9
	V777M	4		
	V842I	31	4	2
	T862A	11	1	2
	H878Y	2		1
	R896C	1		
	R896H	1		
Truncation[a]		N=5		
	A1232fs*25+	2	1	
	G1189fs*9	1		
	P1170fs*88+	1		
	Q1136fs*5	1		
Indel		N=1		
	P780_Y781insGSP			1
Deletion exon 16		N=2		
	Splice site 1899-59_1945del106			1
	Deletion exon 16			1

ERBB3

Mutation Type	Alteration	Count	Cases with Multiple SV
Missense		N=154	
ECD			
	M60K	7	1
	M91I	1	
	R103H	2	1
	V104L	17	
	V104M	35	2
	N126K	1	
	A232V	17	2
	A245V	2	
	R258H	2	
	G284R	26	1
	D297Y	14	
	K329E	4	
	E332K	3	
	T355A	1	
	T355I	1	1
	Y464C	1	
	G582V	1	1
KD		N=13	
	S846I	5	1
	E928G	8	2
Other		N=5	
	A1023T	1	1
	S1049G	1	
	P1212S	3	

Abbreviations: ECD, extracellular domain; JM, juxtamembrane region; KD, kinase domain; mCRC, metastatic colorectal cancer; SV, short variant; TM, transmembrane domain.

[a] Frameshift alterations are as follows: first amino acid and position changed, fs* to note variant type and termination codon, position of termination codon relative to first amino acid changed. A plus sign (+) indicates that no termination codon was observed in the new frame before the end of the original coding sequence.

termination codon of the protein (amino acid 1255). No termination codon is observed in this particular sequence.

Tumor mutational burden (TMB) was determined on 0.83 to 1.14 megabase (Mb) of sequenced DNA using a mutation burden estimation algorithm that, based on the genomic alterations detected, extrapolates to the genome as a whole.[45] For purposes of mutation burden estimation, all coding short variant alterations (SV) (base substitutions and indels), including synonymous alterations, are counted. Subtracted from this number are functionally oncogenic or germline alterations, as defined below. Germline alterations are those listed in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP), those with ≥ 2 counts in the ExAC database (http://exac.broadinstitute.org), or those predicted by a somatic-germline zygosity algorithm to be germline in the specimen being assessed (unpublished data). Functionally oncogenic mutations are those occurring as known somatic alterations in the COSMIC database (http://cancer.sanger.ac.uk/cosmic) or with likely functional status (disruptive alterations in tumor suppressor genes). Finally, to calculate the mutation burden per Mb (mut/Mb), the total number of relevant mutations is divided by the coding

region target territory of the test (0.83 Mb for version 1 and 1.14 Mb for version 2). High TMB was defined in this study as ≥20 mutations/Mb of sequenced DNA; a subset of those cases are designated as hypermutated and have ≥50 mutations/Mb.

The 114 loci used to evaluate microsatellite instability (MSI) status were selected from a total set of 1897 loci that have adequate coverage on both the version 1 and version 2 bait sets. Among the 1897 microsatellites, the 114 that maximized variability between samples were chosen. Each chosen locus was intronic and had hg19 reference repeat length of 10-20 base pairs (bp). This range of repeat lengths was chosen such that the microsatellites are long enough to produce a high rate of DNA polymerase slippage, while short enough such that they are well within the 49-bp read length of next-generation sequencing to facilitate alignment to the human reference genome. Using the 114 loci, for each sample we calculated the repeat length in each read that spans the locus. We recorded the means and variances of repeat lengths across the reads, forming 228 data points per sample. In a large training set of data from clinical specimens, we then used principal components analysis to project the 228-dimension data onto a single dimension (the first principal component) that maximizes the data separation, producing a next-generation sequencing-based "MSI score." There was no need to extend beyond the first principal component, because it explained approximately 50% of the total data variance, whereas none of the other principal components explained >4% each. Ranges of the MSI score were assigned MSI-high, MSI ambiguous, or microsatellite stable. MSI-low calls are not made because there was no gold-standard test set, but we presume such samples would significantly overlap with the MSI-ambiguous category reported here. For samples with low coverage (<250 times the median), a status of MSI unknown is assigned.

RESULTS

The comprehensive genomic profiles from a series of 8887 consecutive cases of mCRC, including both colonic adenocarcinomas (7599 cases; 85.5%) and rectal adenocarcinomas (1288 cases; 14.5%), were evaluated for clinically relevant genomic alterations (Table 1, Supporting Information Table 3) (Figs. 1A-1E). The distribution of patients with CRC harboring *ERBB2/3* alterations was 45% females and 55% males. The median age was 56 years (range, 8-96 years). Samples positive for *ERBB2* amplification and *ERBB3* alterations tended to be from younger patients: median age of 54 years (range, 22-88 years) for patients with *ERBB2* amplification; 54 years (range, 14-83 years) for patients with *ERBB3* alterations; 53 years (range, 46-80 years) for patients with both *ERBB2* and *ERBB3* alterations; compared with 59 years (range, 31-79 years) for patients with only *ERBB2* SV.

A total of 569 samples (6.4%) harbored alterations affecting *ERBB2* (429 cases; 4.8%), *ERBB3* (148 cases; 1.7%), or both *ERBB2* and *ERBB3* (8 cases; 0.1%) (Fig. 2A). The *ERBB2*-positive mCRC cases featured samples with *ERBB2* amplification only (251 cases; 58.5%), a SV sequence alteration in *ERBB2* (135 cases; 31.5%) (Fig. 2B), or cooccurring SV and amplification alterations in *ERBB2* (35 cases; 8.2%). The 8 samples with cooccurring mutations in *ERBB2* and *ERBB3* (0.1%) harbored only ERBB SV. No activating *ERBB2* genomic rearrangements were identified. A total of 189 *ERBB2* SV were detected across 178 mCRC samples, represented by 33 different alterations (Table 2) (Fig. 2B). Of these, 180 of 189 (95.8%) were of known activating alterations. The vast majority of *ERBB2* SV detected encode missense alterations, although 1 instance of an exon 20 insertion (P780_Y781insGSP) and 2 alterations expected to delete exon 16 were detected (Table 2). The remaining 4 alterations were frameshift mutations located at the C-terminus of the protein that may affect regulation of HER2 (Table 2) (Fig. 2B). Of the 148 samples harboring *ERBB3* alterations, 2 had amplification only (1.4%) and 138 harbored only SV in *ERBB3* (93.2%) (Table 2) (Fig. 2A). Of these SV, 110 of 154 cases (71.4%) were of known activating alterations,[26] with the remaining 44 instances distributed among suspected activating alterations and somatically recurrent cancer-related mutations (Table 2) (Fig. 2B).[43]

Amplification of *ERBB2* was defined as ≥ 5 copies of *ERBB2* above the average ploidy of the tumor sample. Of the 286 samples with amplification of *ERBB2*, 284 of 286 had focal amplification (defined as ≤ 20 Mb) of the region surrounding *ERBB2* and 2 of 286 samples harbored amplification of a region >20 Mb (45.6 Mb and 55.8 Mb, respectively). The size of the amplified segment containing *ERBB2* for each sample is reported in Supporting Information Table 4.

The median TMB in the *ERBB*-positive mCRC cases was 4.5 mut/Mb (*ERBB2*) overall, 3.6 mut/Mb for cases with amplification only, 6.3 mut/Mb for cases with SV only, and 3.8 mut/Mb for cases with both SV and amplification (Table 1). This is compared with 5.4 mut/Mb in *ERBB3*-mutated samples and 3.8 mut/Mb for wild-type mCRC. A TMB score ≥ 20 mut/Mb was significantly more common in the mCRC with *ERBB* SV, with 24.5% of *ERBB2*-mutated, 20.9% of *ERBB3*-mutated, and 75% (6 of 8) of *ERBB2/3*-mutated mCRC having

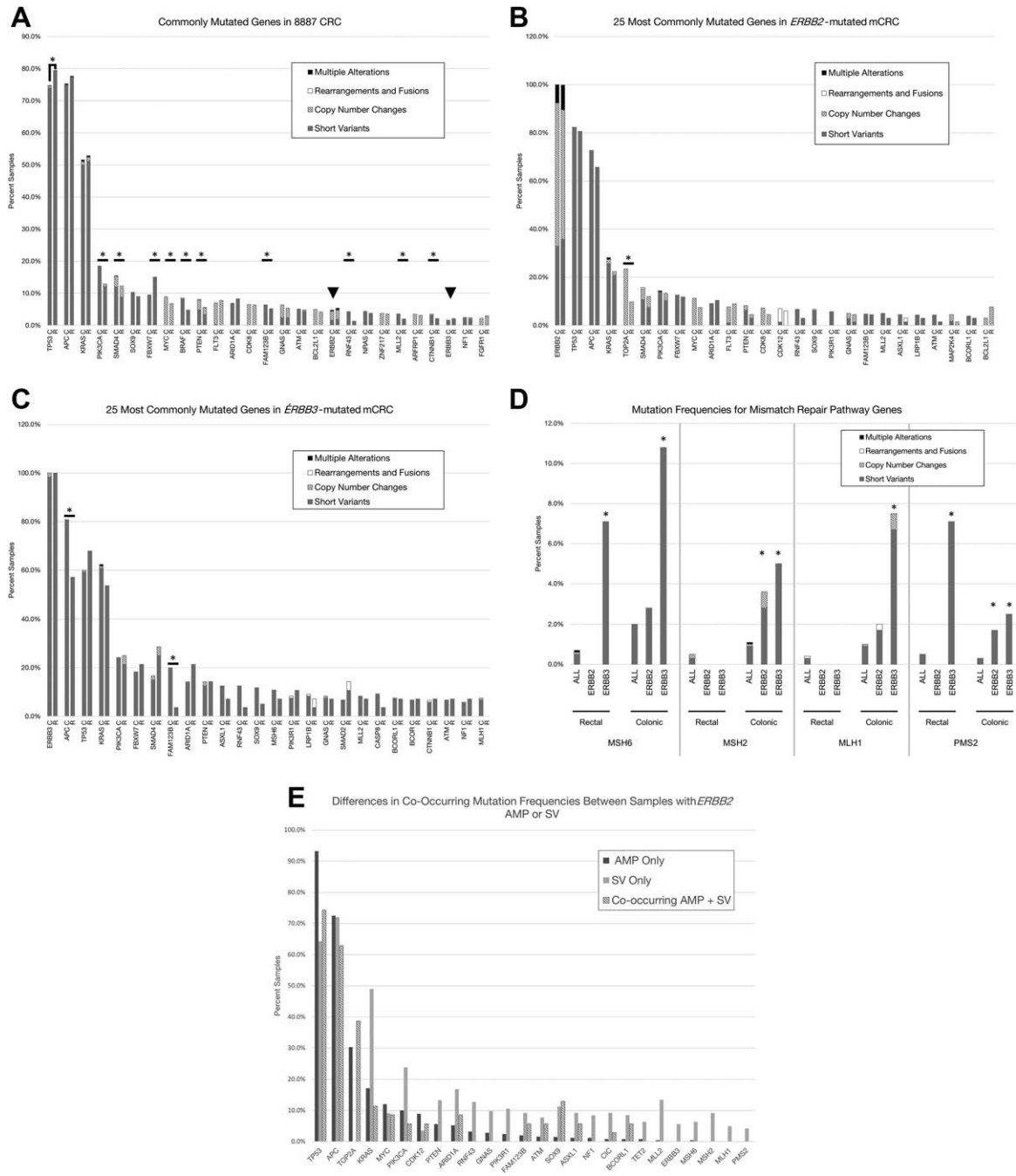

Figure 1. Genes commonly altered in metastatic colorectal cancer (mCRC) and cooccurrence with mutations in *ERBB2* or *ERBB3*. Statistically significant differences in mutation frequencies ($P<.05$) by the Fisher exact text are indicated with an asterisk; differences without an asterisk were not statistically significant. (A) The frequency of gene mutations in 8887 colonic (denoted by C) and rectal (denoted by R) adenocarcinomas. (B) Genes coaltered with *ERBB2* in colonic and rectal mCRCs. (C) Genes coaltered with *ERBB3* in colonic adenocarcinomas. (D) Mutation frequencies for genes in the mismatch repair pathway in all samples, *ERBB2*-mutated, and *ERBB3*-mutated samples for colonic and rectal mCRC. Statistically significant ($P<.05$) differences between *ERBB2*-mutated or *ERBB3*-mutated and nonmutated samples are highlighted with an asterisk. (E) Differences in mutation frequencies among samples with *ERBB2* amplification (AMP) only, short variants (SV) only, or cooccurring AMP and SV (the statistical significance of observations illustrated in Figure 1E is reported in Table 3). MLH1 indicates MutL homolog 1; *MSH2*, mutS homolog 2*; MSH6*, mutS homolog 6.

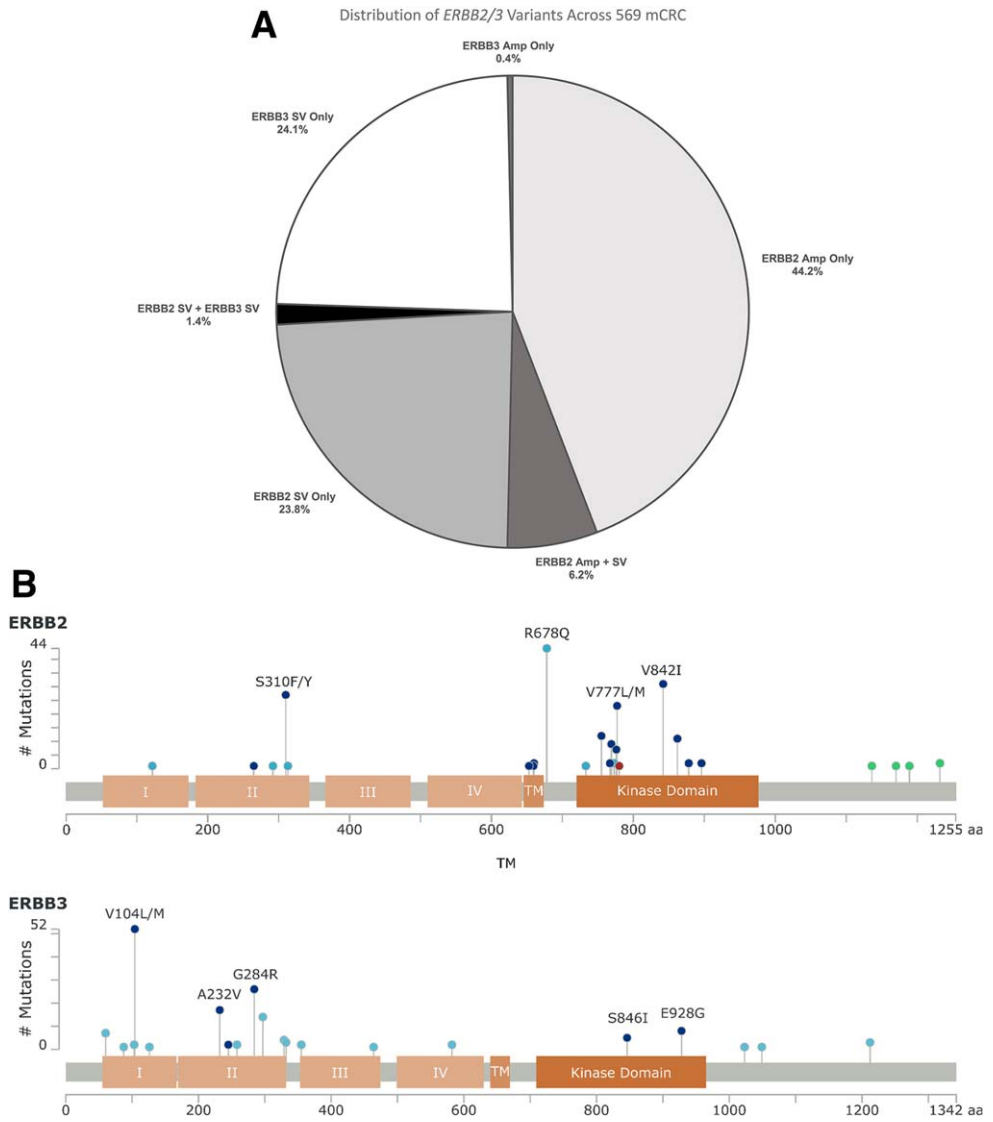

Figure 2. (A) Distribution of the *ERBB2/3* variants in 569 metastatic colorectal cancer (mCRC) cases. (B) Alterations most commonly observed in (*Top*) *ERBB2* and (*Bottom*) *ERBB3*. Shown here are the extracellular (I-IV), transmembrane (TM), and kinase (KD) domains of human epidermal growth factor receptor 2 (HER2) and HER3. Dark blue dots represent known activating missense alterations, whereas light blue dots are missense mutations suspected to be activating or recurrent in cancer. Green dots represent truncating frameshift alterations that are expected to remove a regulatory phosphorylation site from the C-terminus of HER2. Amp indicates amplification; SV, short variants.

high TMB (<<.0001) (Table 1). By contrast, it is significant that none of the samples with *ERBB2* amplification were high TMB ($P<.0005$). A significant number of *ERBB2*-mutated and *ERBB3*-mutated mCRC were MSI-high, at 17.2% to 18.3%, compared with 6.9% of wild-type mCRC ($P<.05$). In addition, 3 of 5 of the *ERBB2/3*-mutated mCRC (60%) tested for MSI demonstrated high levels of MSI ($P<.004$); whereas none of the samples with *ERBB2* amplification did ($P<.005$).

A survey of the genomic alterations observed in the colonic and rectal mCRC cases are shown in Figures 1A to 1E and Table 3. The genes most frequently coaltered with *ERBB* alterations are shown in Figure 1B to 1E. Similar to *ERBB* wild-type samples, tumor protein p53 (*TP53*), adenomatous polyposis coli (*APC*), and *KRAS* were the most frequently altered genes in *ERBB*-mutated samples (Figs. 1B-1C) (Table 3). However, the frequency of *KRAS* alterations was significantly reduced in *ERBB2* amplification samples

TABLE 3. Significant Differences in Gene Mutation Frequencies Between *ERBB2/3*-Mutated Colonic and Rectal mCRC

Colonic						
Gene	All	*ERBB2*+	Fisher Exact Test *P*[a]	*ERBB3*+	Fisher Exact Test *P*[a]	Fisher Exact Test *P*[b]
TP53	74.9%	82.3%	<.001	60.0%	<.001	<.00001
APC	75.3%	72.7%	NS	80.8%	NS	NS
KRAS	51.6%	28.2%	<.00001	62.5%	<.02	<.00001
PIK3CA	18.5%	14.4%	<.05	24.2%	NS	<.02
SMAD4	15.4%	15.7%	NS	16.7%	NS	NS
SOX9	10.2%	6.7%	<.05	11.8%	NS	NS
FBXW7	9.4%	12.7%	<.05	18.3%	<.01	NS
MYC	9.0%	11.3%	NS	4.2%	NS	<.02
BRAF	8.6%	3.6%	<.001	5.0%	NS	NS
PTEN	8.1%	8.3%	NS	14.2%	<.05	NS
ARID1A	6.8%	9.1%	NS	14.2%	<.01	NS
FAM123B	6.4%	4.7%	NS	20.0%	<.00001	<.00001
BCL2L1	5.0%	3.1%	NS	0.0%	<.05	NS
RNF43	4.4%	6.7%	<.05	12.5%	<.001	NS
NRAS	4.3%	1.7%	<.01	3.3%	NS	NS
MLL2	3.6%	5.0%	NS	8.3%	<.02	NS
NF1	2.5%	3.6%	NS	5.8%	<.05	NS
TOP2A	1.2%	23.5%	<.00001	2.4%	NS	<.00001
CDK12	1.0%	7.0%	<.00001	3.3%	<.05	NS
PIK3R1	3.6%	5.8%	<.05	8.3%	<.02	NS
ASXL1	3.6%	4.5%	NS	12.5%	<.0001	<.01
LRP1B	3.6%	4.4%	NS	9.2%	<.01	NS
MAP2K4	2.7%	4.4%	<.05	3.3%	NS	NS
BCORL1	2.2%	3.9%	<.05	7.5%	<.01	NS
MSH6	2.0%	2.8%	NS	10.8%	<.00001	<.0001
MLH1	1.1%	1.9%	NS	7.5%	<.00001	<.01
MSH2	1.1%	3.6%	<.001	5.0%	<.01	NS
PMS2	0.3%	1.7%	<.001	2.5%	<.01	NS
Rectal						
Gene	All	*ERBB2*+	Fisher Exact Test *P*[a]	*ERBB3*+	Fisher Exact Test *P*[a]	Fisher Exact Test *P*[b]
TP53	79.6%	80.6%	NS	67.9%	NS	NS
APC	77.7%	65.7%	<.05	57.1%	<.02	NS
KRAS	53.0%	22.4%	<.00001	53.6%	NS	<.01
PIK3CA	12.9%	13.4%	NS	25.0%	NS	NS
SMAD4	12.3%	11.9%	NS	28.6%	<.02	NS
SOX9	8.9%	0.0%	<.02	5.0%	NS	NS
ARID1A	8.2%	10.4%	NS	21.4%	<.05	NS
TOP2A	0.6%	9.8%	<.00001	0.0%	NS	NS
CDK12	0.6%	6.1%	<.001	0.0%	NS	NS
PIK3R1	2.3%	0.0%	NS	10.7%	<.05	<.05
BCORL1	0.9%	3.0%	NS	7.1%	<.05	NS
SMAD2	3.3%	6.0%	NS	14.3%	<.02	NS
FAM123B	5.2%	4.5%	NS	3.6%	NS	NS

Abbreviations: APC, adenomatous polyposis coli; *ARID1A*, AT-rich interaction domain 1A; *ASXL1*, additional sex combs-like 1; *BCL2L1*, Bcl-2-like 1; *BCORL1*, BCL6 corepressor-like 1; *CDK12*, cyclin-dependent kinase 12; *FBXW7*, F-box/WD repeat-containing protein 7; *LRP1B*, low-density lipoprotein receptor-related protein 1B; *MAP2K4*, mitogen-activated protein kinase 4; mCRC, metastatic colorectal cancer; *MLH1*, MutL homolog 1; *MLL2*, mixed linage leukemia gene 2; *MSH2*, mutS homolog 2; *MSH6*, mutS homolog 6; *NF1*, neurofibromatosis type 1; NS, not significant; *PIK3CA*, phosphatidylinositol 3-kinase; *PIK3R1*, phosphoinositide-3-kinase regulatory subunit 1; *PTEN*, phosphatase and tensin homolog; *RNF43*, ring finger protein 43; *SOX9*, SRY-box 9; *TOP2A*, topoisomerase (DNA) II alpha; *TP53*, tumor protein p53.

[a] Significance values for the difference in frequency between all mCRC and *ERBB2*-mutated or *ERBB3*-mutated samples, respectively.

[b] Significance values for the difference in frequency between *ERBB2*-mutated and *ERBB3*-mutated samples.

(17.1%) compared with all mCRC (51.8%) samples, *ERBB2* SV only (49.0%), or *ERBB3*-mutated mCRC (60.8%) ($P<.00001$). *KRAS* alterations were much more likely to cooccur with SV in *ERBB2* than amplification ($P<.00001$). Similar results were observed for *NRAS* (Table 3).

TABLE 4. Mutation Frequency Differences Between Samples With *ERBB2* Amplification or Short Variants

	ERBB2 Alteration(s)			
	AMP Only	SV Only	Cooccurring AMP and SV	Fisher Exact Test *P*[a]
TP53	93.2%	64.3%	74.3%	<.00001
APC	72.5%	72.0%	62.9%	NS
TOP2A	30.3%	0.0%	38.7%	<.00001
KRAS	17.1%	49.0%	11.4%	<.00001
MYC	12.0%	9.1%	8.6%	NS
PIK3CA	10.0%	23.8%	5.7%	<.001
CDK12	8.9%	3.5%	5.7%	NS
PTEN	5.6%	13.3%	0.0%	<.02
ARID1A	5.2%	16.8%	8.6%	<.001
RNF43	3.2%	12.7%	0.0%	<.001
GNAS	2.8%	9.8%	0.0%	<.01
PIK3R1	2.4%	10.5%	0.0%	<.001
FAM123B	2.0%	9.2%	5.7%	<.01
ATM	1.6%	7.7%	5.7%	<.01
SOX9	1.5%	11.2%	12.9%	<.001
ASXL1	1.2%	9.2%	5.7%	<.001
NF1	1.2%	8.4%	0.0%	<.001
CIC	0.8%	9.2%	2.9%	<.0001
BCORL1	0.8%	8.5%	5.7%	<.001
TET2	0.8%	6.3%	0.0%	<.01
MLL2	0.4%	13.4%	0.0%	<.00001
ERBB3	0.0%	5.6%	0.0%	<.001
MSH6	0.4%	6.3%	0.0%	<.001
MSH2	0.0%	9.1%	0.0%	<.00001
MLH1	0.0%	4.9%	0.0%	<.001
PMS2	0.0%	4.2%	0.0%	<.01
Median TMB, mut/Mb	3.6	6.3	3.8	
TMB range, mut/Mb	0-16.2	0-230.6	0-10.1	
High MSI	0/172 (0.0%)	15/88 (17.0%)	0/24 (0.0%)	<.00001

AMP, amplification; APC, adenomatous polyposis coli; *ARID1A*, AT-rich interaction domain 1A; *ASXL1*, additional sex combs-like 1; *ATM*, ataxia-telangiectasia mutated; *BCORL1*, BCL6 corepressor-like 1; *CDK12*, cyclin-dependent kinase 12; *CIC*, Capicua transcriptional repressor; *MLH1*, MutL homolog 1; *MLL2*, mixed linage leukemia gene 2; *MSH2*, mutS homolog 2; *MSH6*, mutS homolog 6; MSI, microsatellite instability; mut/Mb, mutation burden per megabase; *NF1*, neurofibromatosis type 1; NS, not significant; *PIK3CA*, phosphatidylinositol 3-kinase; *PIK3R1*, phosphoinositide-3-kinase regulatory subunit 1; *PTEN*, phosphatase and tensin homolog; *RNF43*, ring finger protein 43; *SOX9*, SRY-box 9; SV, short variants; TMB, tumor mutational burden; *TET2*, Tet methylcytosine dioxygenase 2 ; *TOP2A*, topoisomerase (DNA) II alpha; *TP53*, tumor protein p53.

[a] Significance of the difference between cases harboring only amplification or only short variant alterations in *ERBB2* calculated by the Fisher exact test.

Conversely, alterations in *TP53* were far more common in *ERBB2* amplification samples (86.7%-91.7%) compared with *ERBB2* SV samples (64.4%-71.9%), ERBB2 SV plus amplification samples (74.1%-75.0%), *ERBB3*-mutated samples (60.0%-67.9%), or the cohort in general (74.9%-79.6%). Coamplification of topoisomerase (DNA) II alpha (*TOP2A*), which is colocalized with *ERBB2* on chromosome 12, was found in 34.5% of samples harboring *ERBB2* amplification but not in any samples with only *ERBB2* SV (Table 4). An example of cooccurring amplification of *ERBB2* and *TOP2A* is shown in Figure 3. Alterations in *BRAF*, phosphatidylinositol 3-kinase (*PIK3CA*), or SRY-box 9 (*SOX9*) were less common in *ERBB2* amplification samples, whereas alterations in *CDK12* and ring finger protein 43 (*RNF43*) were more likely (Table 3) (Table 4).

Significant increases in mutation frequencies in *ERBB3*-mutated samples were noted for *FAM123B* (20.0% vs 4.7-6.4%; $P<.00001$) and genes responsible for mismatch repair (mutS homolog 6 [*MSH6*], mutS homolog 2 [*MSH2*], MutL homolog 1 [*MLH1*], and *PMS2*) (Table 4) (Fig. 1D). Alterations in *ASXL1, LRP1B, MLL2, BCORL1*, and phosphoinositide-3-kinase regulatory subunit 1 (*PIK3R1*) were more common in *ERBB3*-mutated colonic mCRC, whereas *SMAD2* and *SMAD4* were more often altered in *ERBB3*-mutated rectal samples (Table 4).

The frequency of cooccurring mutations in other genes also differed slightly between the colonic and rectal mCRC cases, as shown in Figures 1A to 1C. In the context of *ERBB2*, the only significant difference in mutation frequency between colonic and rectal samples was for amplification of *TOP2A*, which was more common in colonic mCRC (23.5% vs 9.8%) ($P<.05$). For *ERBB3*, there were striking differences in mutation frequencies for *APC* (80.8% for colonic vs 57.1% for rectal; $P<.02$) and *FAM123B* (20.0% for colonic vs 3.6% for rectal; $P<.05$).

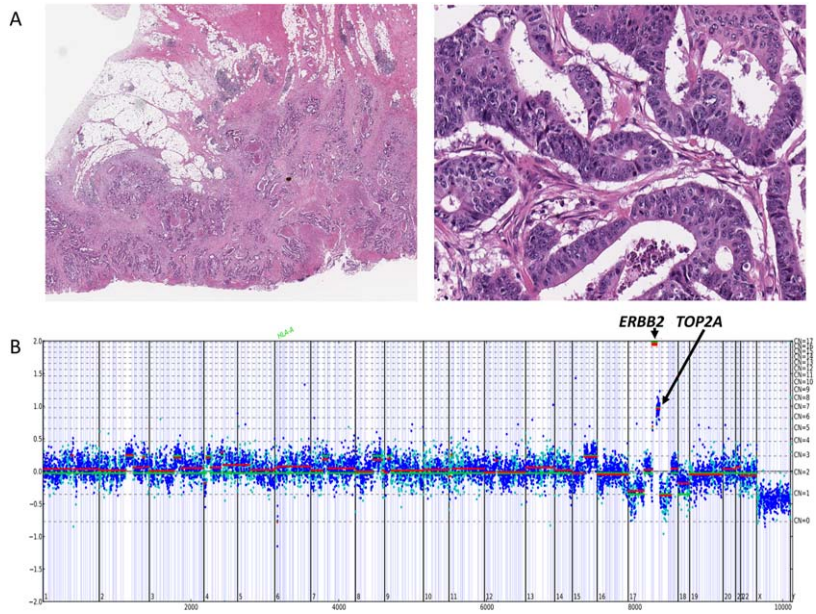

Figure 3. (A) Moderately differentiated adenocarcinoma of the colon in a 72-year-old white man. The tumor invaded through the colon wall and involved numerous pericolonic lymph nodes (pathologic classification T3N2A). The patient rapidly developed stage IV disease. (B) The copy number plot below the histologic images demonstrates extremely high-level amplification of *ERBB2* at 60 copies, associated with lower level coamplification of topoisomerase (DNA) II Alpha (*TOP2A*) at 7 copies. Using comprehensive genomic profiling, this metastatic colorectal cancer also harbored base substitutions in *KRAS* (G12D), F-box/WD repeat-containing protein 7 (*FBXW7*) (R479Q), adenomatous polyposis coli (*APC*) (Q1367*), SRY-box 9 (*SOX9*) (D274fs*22), and tumor protein p53 (*TP53*) (C275W).

An example of *ERBB2*-amplified mCRC responding to anti-HER2 targeted therapy is shown in Figure 4.[46] This widely disseminated rectal mCRC in a 39-year-old woman that was refractory to systemic chemotherapy and multiple metastasectomies responded to a trastuzumab-based regimen. In case Colonic mCRC 220, a 72-year-old woman with CRC that was metastatic to the liver, *ERBB2* was amplified to 163 copies (Fig. 5). Also present were the SV alterations *FBXW7* M118fs*52, *TP53* splice site 672G>A, *APC* R1450*, and *FAM123B* R531*. This tumor responded to a combination of trastuzumab and lapatinib for 6 months after prior failure of 4 separate lines of cytotoxic chemotherapy.

DISCUSSION

In December 1998, the simultaneous approvals of the anti-HER2 targeting antibody trastuzumab and the slide-based IHC test to select patients for therapy ushered in the era of personalized medicine for solid tumors. The following 15 years then saw 2 major evolutions in anti-HER2 therapies: 1) the development and approval of oral anti-HER2 small molecule kinase inhibitors and additional anti-HER2 antibody therapeutics[47]; and 2) the expanded indication from breast cancer to upper gastroesophageal carcinomas.[48] The expanded use of anti-HER2 drugs was coordinated by slide-based tests including IHC, FISH, and chromogenic in situ hybridization (CISH).[4] Additional approaches to detect increased HER2 activity were evaluated, such as measuring HER2 mRNA expression levels, but failed to achieve broad clinical usefulness, possibly due to technical limitations.[49] More recently, the comprehensive genomic analysis of DNA extracted from formalin-fixed, paraffin-embedded samples has been used to survey mCRC, breast, and gastroesophageal cancers for genomic alterations affecting *ERBB2* copy number and sequence.[29,30,34,50-52] When combined with the published data from The Cancer Genome Analysis and the COSMIC database, these studies have reported similar frequencies of *ERBB2* amplification and short variant mutation.[34,43,53]

CRC continues to be a major cause of morbidity and mortality worldwide in both developed and, to a lesser extent, underdeveloped countries.[54] In the United States, CRC is the second most prevalent cancer in males at 724,690 cases and the third most prevalent in women with 727,350 cases.[55] The standard-of-care treatment for mCRC using multiagent chemotherapy is effective at slowing the progress of the disease, but long-term remissions are

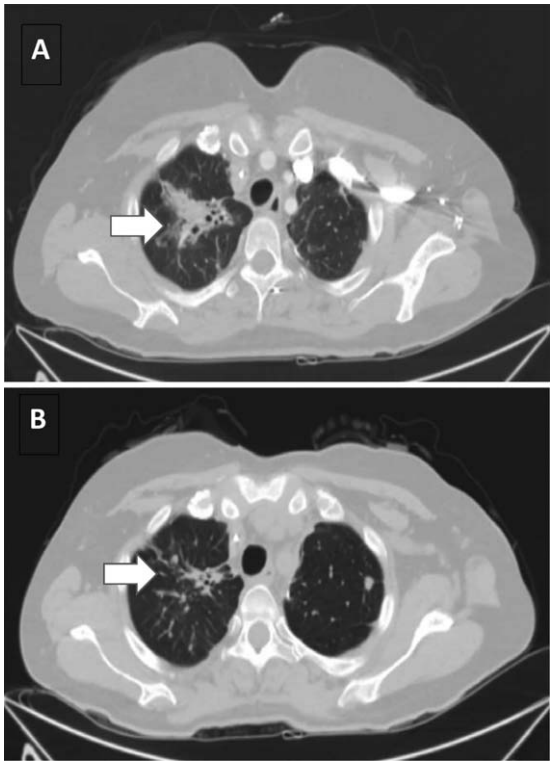

Figure 4. Response of an *ERBB2*-amplified metastatic colorectal cancer (mCRC) to antihuman epidermal growth factor receptor 2 (HER2)-targeted therapy. A 39-year-old woman with a pT3N0 rectal adenocarcinoma developed widespread metastatic disease and was treated with systemic chemotherapy and metastasectomies. The mCRC was found to be *KRAS* wild-type on routine single-gene testing and anti-EGFR therapy with cetuximab was used until disease progression. Comprehensive genomic profiling was performed on a metastasis sample at that time and revealed *ERBB2* amplification at 21 copies and a tumor protein p53 (*TP53*) base substitution. Combination therapy with trastuzumab with a backbone of capecitabine and oxaliplatin was initiated. Treatment with trastuzumab continued for 12 months, after which time the patient's symptoms returned with biomarkers and radiology confirming progressive disease. Representative computed tomography scan image of upper lung metastasis is shown (A) at baseline and (B) after 3 months of trastuzumab and chemotherapy. The arrow indicates significantly regressed tumor burden accounting for improved pulmonary symptoms. The targeted therapy using trastuzumab in combination with cytotoxic chemotherapy maintained a strong response in the patient over a 1-year course of therapy, reducing tumor burden and improving quality of life.

rare and the treatment side effects are often significant.[56,57] This has prompted the development of less toxic targeted therapies for the disease, but progress has been slow.[58-61] Although anti-EGFR antibody therapeutics have been approved for the treatment of mCRC for several years, the use of these agents has been personalized by determining which patients should not be treated due to predicted resistance rather than identifying individuals significantly likely to benefit from treatment.[62] Thus, interest has emerged in finding targeted therapies for which biomarkers can positively predict patient benefit from therapy.[58-61]

Based on the current and previously published studies, *ERBB2* has now emerged as an important target in mCRC. Given the high incidence worldwide of mCRC, the nearly 5% frequency of *ERBB2* genomic alterations makes this an attractive target for future regulatory approval of anti-HER targeted therapies. Two basic strategies for targeting HER2 in mCRC have been taken: 1) targeting HER2 as the primary driver of the disease when appropriate; and 2) attempting to overcome the resistance to other targeted therapies mediated by *ERBB2* genomic alterations.[34] Initial studies targeting *ERBB2* in mCRC focused exclusively on *ERBB2*-amplified cases detected by either FISH or direct sequencing methods.[31,36,63,64] Figure 4 provides an example of an *ERBB2*-amplified mCRC vigorously responding to anti-HER2 targeted therapy.[46] Various levels of success have been achieved targeting *ERBB2* amplification in CRC, and use of both antibody and small molecule treatments has been described.[31,36,46,63-65] To our knowledge, tyrosine kinase inhibitors such as afatinib and lapatinib have yielded limited clinical efficacy as monotherapies in patients with mCRC, suggesting antibody therapeutics or combination therapies may be more beneficial in this tumor type.[66-68] In one report of a widely disseminated, *ERBB2*-amplified mCRC, various combinations of trastuzumab, trastuzumab-DM1 and pertuzumab achieved prolonged patient response and disease control.[69]

Two effective therapeutic options have emerged in the management of *ERBB2*-amplified colorectal cancers. The HERACLES trial[36] investigated a combination of the *ERBB2*-binding monoclonal antibody, trastuzumab, and the *ERBB* tyrosine kinase inhibitor lapatinib. Of 27 heavily pretreated patients with CRC with *ERBB2* amplification, 30% of patients achieved an objective response and 44% achieved disease stabilization. One patient experienced a complete response. Patients with an *ERBB2* copy number > 9.45 derived a significantly better outcome than those with lower levels of amplification. In the current study, 86.9% of samples with *ERBB2* amplification had ≥ 10 copies of the gene.

Another emerging option for this population consists of the combination of trastuzumab plus pertuzumab, which allows for a more effective inhibition of *ERBB2/ERBB3* signaling. In a preliminary analysis, the MyPathway trial reported a response rate of 37.5% with this combination in heavily pretreated patients with CRC with *ERBB2* amplification.[41] The intergroup is in the process of activating a randomized clinical trial of trastuzumab

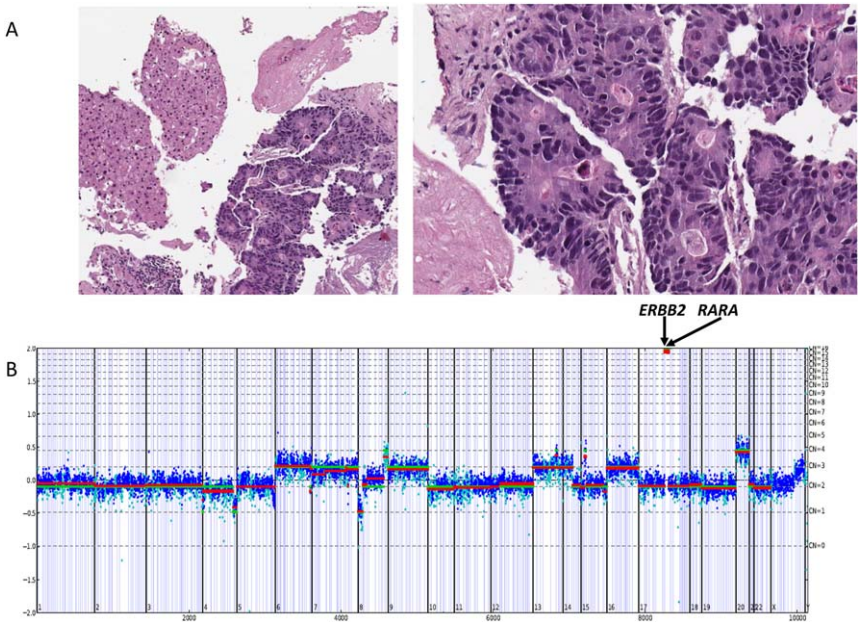

Figure 5. (A) Metastatic colorectal cancer to the liver in a 72-year-old woman whose tumor had progressed after 4 separate lines of chemotherapy. (B) Comprehensive genomic profiling revealed both ERBB2 (163 copies) and retinoic acid receptor alpha (RARA) (35 copies) amplification as well as multiple untargetable short variant genetic alterations. This tumor responded clinically for 6 months to a combination of trastuzumab and lapatinib, with a significant decrease in serum carcinoembryonic antigen levels.

plus pertuzumab in comparison with cetuximab plus irinotecan in the second-line/third-line treatment of *ERBB2*-amplified CRCs (SWOG S1613).

Targeting activating *ERBB2* mutations in CRC is less well-defined clinically. Based on patient-derived tumor xenograft studies, these tumors appear more resistant to HER2-targeting monoclonal antibodies and are more sensitive to the combinations of trastuzumab plus pan-ERBB or HER2-specific tyrosine kinase inhibitors.[70]

An additional 1.7% of mCRCs harbor alterations in the HER2 dimerization partner HER3, which may represent a second group of patients for whom HER2-targeted treatments would be relevant. Indeed, clinical reports of activity of *ERBB2* tyrosine kinase inhibitors with or without trastuzumab have already been reported in breast and urothelial cancers with *ERBB3* mutations.[71,72]

Mutations affecting HER2 or HER3 can drive downstream processes impacting proliferation and invasiveness and resistance to apoptosis, and have emerged as potential therapy targets.[12-18,26,73] The distribution of kinase versus extracellular domain mutations appears to vary by tumor type. For breast cancer, HER2 kinase domain mutations are more common, whereas in urinary bladder cancer, extracellular domain mutations predominate.[3] Some tumor types also feature specific types of alteration, such as the *ERBB2* insertion mutations commonly identified in non-small cell lung cancers.[3] For mCRC, SV mutations in the *ERBB2* sequence accounted for approximately one-third of all *ERBB2* genomic alterations, with rectal tumors having slightly more SV alterations than colonic lesions (Fig. 1B). Recent studies have further emphasized the potential of targeting *ERBB2* sequence mutations in the absence of *ERBB2* amplification in mCRC using combinations of kinase inhibitors and antibody therapeutics.[36,69,70,74,75] The preliminary success described in these reports have generated further interest in expanding clinical trials to include *ERBB2* SV mutations when there are no copy number changes in *ERBB2*.

In the current study, a variety of SV alterations in *ERRB2* were observed (Fig. 2B), with the vast majority being characterized as activating missense alterations (eg, S310F/Y) or missense mutations alterations that are highly recurrent in cancer (eg, R678Q). Missense alterations were clustered in the extracellular domain, the transmembrane domain, and the kinase domain, with the exception of R678Q, which lies within the juxtamembrane region. In addition, we observed several frameshift alterations predicted to truncate HER2, an exon 20 insertion, and 2 alterations predicted to delete exon 16 and

hyperactive HER2 signaling.[76-78] A similar variety of alterations was observed for *ERBB3*, with the majority affecting the extracellular domain (Fig. 2B). Two recurrent alterations in the kinase domain (S846I and E928G) are located at the dimerization interface and have been shown to increase HER3-induced signaling.[79] In addition, preclinical experiments have shown that activating alterations in HER3 can underlie sensitivity to therapies targeting HER2, such as lapatinib or trastuzumab.[26,28] The wide variety of alterations observed reinforces the clinical usefulness of sequencing technologies that can comprehensively interrogate oncogenic genes in an unbiased fashion.

Preclinical and clinical data have suggested that *ERBB2* amplification in mCRC is associated with a lack of response to the EGFR antibodies cetuximab and panitumumab.[34,80-82] In recent reports, a combination of therapies targeting both EGFR and HER2 was effective in treating tumors with *ERBB2* amplification in preclinical experiments.[81,83,84] Mutations in *KRAS* are widely accepted as predictors of resistance to anti-EGFR antibody therapies in patients with mCRC.[85] In the current study and previous reports,[34,86] *ERBB2* amplification strongly correlated with a lack of *RAS* and *BRAF* alterations. In addition, although we did not observe a difference between rectal and colonic tumors in terms of frequency of *ERBB2* amplifications, other studies have demonstrated a strong correlation between *ERBB2* amplification and left colonic tumors.[31,87] Greater than 5% of left colonic tumors with *RAS* wild-type status will harbor *ERBB2* amplifications and with a resultant relative resistance to anti anti-EGFR therapy. The early identification of these genomic alterations will impact the choice of biological therapies in the management of these patients and/or guide them toward the appropriate HER2-targeting clinical trials.

Both TMB and MSI status were evaluated in the current study. The median TMB was higher in cases with *ERBB2* or *ERBB3* SV, and a greater percentage of cases harboring *ERBB* SV had TMB scores $\geq$20 mut/Mb compared with *ERBB* wild-type or *ERBB2* amplified mCRC (Table 1). High MSI was also found for *ERBB* mCRC, and was significantly enriched in *ERBB3*-mutated mCRC. A significant association also was observed between *ERBB3* mutation and alterations in the genes responsible for mismatch repair (*MSH2, MSH6, MLH1*, and *PMS2*). To the best of our knowledge, this association between *ERBB3* and the DNA mismatch repair pathway has not been previously reported. For patients with mCRC, both MSI high status and high TMB have been associated with responsiveness to checkpoint inhibitor immunotherapies.[88-90] Given the enrichment in mutational load for *ERBB* SV samples, combining immunotherapies with anti-HER2 targeting agents for these patients becomes an intriguing possibility.

The use of comprehensive genomic profiling on such a large cohort of CRC cases allowed us to identify several striking differences in mutation frequencies between colonic and rectal adenocarcinomas (Fig. 1) (Table 3), predominantly in the PI3K/MTOR and WNT/β-catenin pathways. Differences also were observed in the mutation frequencies of genes coaltered with *ERBB2* amplification versus SV (Table 4). CRCs harboring *ERBB2* SV had higher mutation frequencies in the PI3K (*PIK3CA, PTEN, PIK3R1*, and *NF1*), mismatch repair (*MSH6, MSH2, MLH1*, and *PMS2*), and Wnt (*RNF43, SOX9*, and *FAM123B*) pathways, among others. It remains to be determined whether the high MSI found in a significant percentage of cases with only *ERBB2* SV (17%) is one mechanism underlying these associations. *TOP2A* amplification was frequently observed within the context of *ERBB2* amplification; *TOP2A* is located near *ERBB2* on chromosome 17.

Although more often observed in breast and upper gastrointestinal carcinomas, for which anti-HER2 therapies currently are approved indications, the frequency of *ERBB* genomic alterations in mCRC (6.4%) is nonetheless significant. It is important to note that nearly one-third of *ERBB2*-altered mCRCs harbor SV alterations only, which are not detectable by routine IHC and FISH testing. Given the successful use of anti-HER2 therapies to treat *ERBB2*-driven mCRC in case studies and ongoing clinical trials, HER2-targeted therapies may one day become approved precision treatments for patients with mCRC.

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AUTHOR CONTRIBUTIONS

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and **James Sun**. Formal analysis and investigation: **Jeffrey S. Ross**, and **Laurie M. Gay**. Methodology and software: **David Fabrizio, Garrett Frampton**, and **James Sun**. Supervision: **Jeffrey S. Ross, Siraj M. Ali, Julia A. Elvin, Vincent A. Miller**, and **Philip J. Stephens**.

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