Repurposing EGFR Inhibitor Utility in Colorectal Cancer in Mutant APC and TP53 Subpopulations

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Abstract

Background: EGFR is a major therapeutic target for colorectal cancer. Currently, extended RAS/RAF testing identifies only nonresponders to EGFR inhibitors (EGFRi). We aimed to develop a mutation signature that further refines drug-sensitive subpopulations to improve EGFRi outcomes.

Methods: A prespecified, 203-gene expression signature score measuring cetuximab sensitivity (CTX-S) was validated with two independent clinical trial datasets of cetuximab-treated patients with colorectal cancer (n = 44 and n = 80) as well as an in vitro dataset of 147 cell lines. The CTX-S score was then used to decipher mutated genes that predict EGFRi sensitivity. The predictive value of the identified mutation signature was further validated by additional independent datasets.

Results: Here, we report the discovery of a 2-gene (APC+TP53) mutation signature that was useful in identifying EGFRi-sensitive colorectal cancer subpopulations. Mutant APC+TP53 tumors were more predominant in left- versus right-sided colorectal cancers (52% vs. 21%, P = 0.0004), in microsatellite stable (MSS) versus microsatellite instable (MSI) cases (47% vs. 2%, P < 0.0001), and in the consensus molecular subtype 2 versus others (75% vs. 37%, P < 0.0001). Moreover, mutant APC+TP53 tumors had favorable outcomes in two cetuximab-treated patient-derived tumor xenograft (PDX) datasets (P = 0.0277, n = 52; P = 0.0008, n = 98).

Conclusions: Our findings suggest that the APC and TP53 combination mutation may account for the heterogeneity of EGFRi sensitivity and provide a rationale for refining treated populations. The results also suggest addition of APC+TP53 sequencing to extended RAS/RAF testing that may directly increase the response rates of EGFRi therapy in selected patients.

Impact: These findings, if further validated through clinical trials, could also expand the utility of EGFRi therapies that are currently underutilized.

Introduction

Two well-characterized EGFR inhibitors (EGFRi; cetuximab, panitumumab) are FDA approved as first- and second-line targeted therapies for metastatic colorectal cancer (mCRC; refs. 1–8). Despite approval, utilization has been modest, primarily because of drug restriction to the wild-type (WT) RAS subpopulation. Early colorectal cancer clinical trial studies involving cetuximab/panitumumab, either as monotherapies or as combination therapies, reported that a statistically significant drug response was generally observed in WT KRAS patients—but not in MUT KRAS patients (5, 8). On the other hand, despite selection, about half of patients with a WT KRAS still fail to respond to EGFRi treatments (9, 10), suggesting that additional genes, beyond KRAS, may negatively contribute to EGFRi response. Recently, mutations in NRAS and BRAF were reported to account for EGFRi therapy resistance in some WT KRAS colorectal cancers (1–3). More recently, left-sided colorectal cancers have been reported to be more favorably associated with response to cetuximab/panitumumab than right-sided tumors, as indicated by increased response rate (RR), better progression-free survival (PFS), and/or overall survival (OS; refs. 6, 11–13). A molecular basis of the laterality of anti-EGFR sensitivity, however, is still poorly understood.

We recently developed a new, robust molecular classification of colorectal cancer to help dissect this heterogeneous disease into five molecular subpopulations to improve treatment strategies (14, 15). This classification complements the recently reported consensus molecular subtypes (CMS) of colorectal cancer that were coalesced from six independent (gene expression) colorectal cancer classification systems (16). We performed an integrated analysis–targeted gene sequencing for 1,321 cancer-related genes, global gene expression, and MSI analyses across a large cohort of human colorectal cancer (n = 468). Among a number of mutated genes identified, striking pairwise, statistically significant, correlations were observed between APC, TP53, KRAS, and BRAF that ultimately suggested a prognostic role for APC (15). On the basis of these results, we hypothesized there might also be a predictive role for APC and other associated genes.

Given the paucity of available clinical trial tissue samples with EGFRi exposure, we elected to use a cetuximab sensitivity (CTX-S) gene expression score as a surrogate for cetuximab response data in our colorectal cancer cohort, TCGA, and other published data.
This approach allowed us to develop a 2-gene "mutation signature" that is strongly correlated with the CTX-S score and can be rapidly translated to the clinic.

**Materials and Methods**

**Datasets of patient samples, cell lines, and PDX models**

We previously analyzed 468 stage I–IV colorectal tumors, with global gene expression data from the surgical specimen, MSI status, and targeted gene sequencing of 1,321 cancer-related genes (14, 15). A cohort of the 468 patients with colorectal adenocarcinoma (including 367 primary lesions from stage I–IV patients and 101 metastatic lesions) was accrued between October 2006 and September 2010, and written informed consent was obtained from participating patients as part of the Total Cancer Care (TCC) project (17). The study was conducted in accordance with recognized ethical guidelines (Declaration of Helsinki, CIOMS, Belmont Report, U.S. Common Rule) and under the approval of the University of South Florida institutional review board (17). Primary and metastatic samples were both included on the basis of our previous work demonstrating a high degree of mutation overlap between matched primary/metastatic samples (18). Here, we further used this large, well-curated clinicogenomics/expression database of colorectal cancer patient samples to carry out mutation ranking analysis by the CTX-S score and other statistical analyses. We identified seven additional independent datasets, from Merck and public resources including Gene Expression Omnibus (GEO) and NCI Genomic Data Commons (GDC), for various validation and correlation analyses. These included WT KRAS colorectal cancer samples (n = 44) selected from the control arm (cetuximab + irinotecan) of a Merck prospective clinical trial (MK0646; ref. 19), a BMS trial cetuximab-treated colorectal cancer patient samples [(n = 80, Khambata-Ford and colleagues (4)], in vitro cetuximab-treated colorectal cancer cell lines [(n = 147, Medico and colleagues (20)], TCGA colorectal cancer patient samples [(n = 624 including 221 DNA-sequenced samples from TCGA (21)], and an additional set of stages I–IV colorectal cancer patients samples [(n = 566, Marisa and colleagues (22)], as well as cetuximab-treated colorectal cancer PDX models [(n = 52, Julien and colleagues (23)] and n = 98, Bertotti and colleagues (24)]. A summary of all eight datasets is given in Table 1, and detailed data description is given in Supplementary Methods and Tables S1–S8.

**Expression signatures (see Supplementary Table S9 for gene lists)**

The **CTX-S score**. A prespecified gene expression signature score that measures CTX-S was initially constructed on the basis of gene expression values from >800 cancer-associated genes, each assessed in a set of 44 WT KRAS colon tumor samples from patients treated with cetuximab monotherapy. Two-hundred and three genes with P < 0.05 by PFS Cox Regression analysis were identified that included 94 UP and 109 DOWN expressed genes based on their association with response or resistance to cetuximab, respectively. CTX-S score was defined as the average expression of the genes in the UP arm minus the average expression of genes in the DOWN arm. CTX-S was prespecified for subsequent analysis of the validation sets. The signature score derivation and validation is summarized in Supplementary Fig. S1 and overall methodology used is similar to as we reported in previous studies (14, 25, 26).

The **18-gene RAS pathway score**. This score was developed to measure the MEK functional output in association with RAS pathway activation (27).

The **64-gene Wnt pathway score**. We previously adopted a set of 64 "consensus" β-catenin (upregulated) genes from a recent study of Herbst and colleagues (28) to assess differential activation of Wnt pathway in APC subgroups of Moffitt colorectal cancers (15). The 64-gene Wnt pathway scores were calculated from the arithmetic mean expression of the 64 genes.

The **24-gene APC–specific Wnt pathway score**. We further selected 24 genes (out of the 64 genes) whose expression was significantly higher in APC-mutated tumors than those with WT APC (P < 0.05 for two-tailed Welch t test; see Supplementary Fig. S2A). The arithmetic mean expression of selected 24 genes is designated as the APC–mutation specific Wnt pathway score, which was validated by TCGA colorectal cancers (n = 221; Supplementary Fig. S2B).

**Statistical analysis**

The statistical approaches used include the following: (i) survival analysis, correlation analysis, and the t test; (ii) mutation ranking analysis of Moffitt 468 colorectal cancers; (iii) Cochrane–Mantel–Haenszel (CMH) test, Barnard test, and distribution analysis; (iv) CMS classification. See Supplementary Methods for detailed description.

The statistical tests used in the article were given unadjusted P values for multiple testing with an α = 0.05 chosen as the significance level, except for the mutation ranking analyses that use the Benjamini and Hochberg FDR method (29). In addition, for the Welch t test in comparison among 7 or 5 MSI/MMC subgroups, those unadjusted P values remaining significant after adjustments for multicomparisons by Holm–Bonferroni method (30) were highlighted by a maroon color. All tests were two-sided unless noted otherwise.

**Results**

**Development and validation of the CTX-S score**

The CTX-S signature score was validated using two independent test sets of cetuximab–treated patients and a test set of cetuximab–treated colorectal cancer cell lines, as summarized in Supplementary Fig. S1. A detailed description of the results is given below:

**Validation set 1 (in vivo)**. The CTX-S score was first validated using 44 colorectal cancer control arm samples and data from a Merck prospective clinical trial (MK0646), a randomized phase II/III study of dalotuzumab (IGF-1R inhibitor) in combination with cetuximab and irinotecan in chemo-refractory, KRAS WT, mCRC (19). In the control arm "C" of MK0646, high IGF-1 expression was shown to be significantly associated with lower response rates to cetuximab + irinotecan and IGF-1 was considered to be a promising biomarker for differential response to anti-IGF1R therapies as well as anti-EGFR therapies (19). Here, we used the MK0646 control arm samples to further test whether the CTX-S signature score could predict positive outcomes to cetuximab + irinotecan therapy. Barnard exact test revealed that the CTX-S score was significantly associated with objective responses (OR; vs. no objective response, P = 0.0048; see Fig. 1A; Supplementary Table S10A). Moreover, Kaplan–Meier (KM) survival
Table 1. List of eight colorectal cancer datasets used

<table>
<thead>
<tr>
<th>Datasets</th>
<th>Samples</th>
<th>Number of samples</th>
<th>Stage</th>
<th>Cetuximab response</th>
<th>Prognosis</th>
<th>Gene expression</th>
<th>MSI status</th>
<th>DNA sequencing</th>
<th>Driver mutations</th>
<th>Primary tumor location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MK0646- PN004 colorectal cancers (19)</td>
<td>Patient tumors</td>
<td>44</td>
<td>IV</td>
<td></td>
<td>PFS, OS OR</td>
<td>Affymetrix</td>
<td>Targeted sequencing</td>
<td>KRAS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. TCGA colorectal cancers—TCGA Nature (2012; ref. 20; NCI GDC)</td>
<td>Patient tumors</td>
<td>624</td>
<td>I, II, III, IV</td>
<td></td>
<td>RNAseq</td>
<td>MS/MSS</td>
<td>Whole-exome sequencing</td>
<td>APC, TP53, KRAS, NRAS, BRAF</td>
<td>Left/right</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Also see Supplementary Tables S1-S8 for detailed data description of individual datasets, respectively.

Abbreviations: MSI (i.e., MSI-high), microsatellite instable tumors; MSS, microsatellite stable tumors; PD, progressed disease; PDX, patient-derived tumor xenograft.
Figure 1.
Validation analysis of the CTX-S signature score in two independent sets of colorectal cancer patient samples derived from clinical trials and one set of in vitro cetuximab-treated colorectal cancer cell lines. A, A waterfall plot of objective response (OR) versus adjusted CTX-S score in MK0646 PN004 WT KRAS colorectal cancers (n = 44; see Supplementary Table S1 for detailed data description). The P value is for Barnard exact test (OR: No OR; see Supplementary Table S10A). B, KM survival (PFS) analysis by higher (>0) versus lower (<0) CTX-S scores was also performed the PN004 colorectal cancers (n = 41). A and B, of 44 colorectal cancers, one sample with PFS of 1 day and two samples with CTX-S scores near 0.00 as shown in A, were excluded from Barnard test and KM analysis. Also see Supplementary Fig. S3 for similar KM analysis on OS. C, KM survival (PFS) analysis by the CTX-S quartile scores was performed in Khambata-Ford and colleagues (2007) cetuximab-treated colorectal cancers (n = 80; ref. 4). D, KM PFS analysis in Khambata-Ford WT KRAS patients (n = 43). Also see Supplementary Tables S10B and S10C for the CMH test showing the significant association of the CTX-S score with improved response (CR/PR and SD). E, Spearman correlation analysis of the CTX-S score with in vitro growth inhibition (%) by 10 μg/mL of cetuximab in Medico and colleagues (2015) cetuximab-treated colorectal cancer cell lines (20). The analysis was performed in all cell lines (n = 147) and WT RAS (KRAS/NRAS) cell lines (n = 77) as well as MSS (n = 87) and MSI (n = 60) cell lines, respectively (see detailed data description in Supplementary Table S3A). The results for the other doses of cetuximab (1, 25, 50, and 100 μg/mL) were similar, as 10 μg/mL had a 0.945 or higher (Pearson) correlation with these doses for in vitro growth inhibition (Supplementary Table S3B).
analysis showed that the CTX-S score was significantly associated with longer PFS; 208 vs. 83 days, HR = 0.34; 95% CI, 0.07–0.51; P = 0.0018; Fig. 1B) and longer OS; 503 vs. 287 days, HR = 0.30; 95% CI, 0.05–0.58; P = 0.0052; Supplementary Fig. S3).

Validation set 2 (in vivo). The CTX-S score was subsequently validated using a second independent, well-characterized, dataset of 80 patients with mCRC prospectively treated with cetuximab mono-therapy from a BMS clinical trial (4). This dataset was initially used to identify EREG and AREG as predictive markers whose high expression was significantly associated with longer PFS in cetuximab-treated patients (4), and later used in a variety of other gene expression classification and validation analyses associated with EGFRi treatment prediction (31–33). Notably, EREG is a member of the CTX-S score “UP genes,” whereas AREG is not included in the gene list of the CTX-S (see Supplementary Table S9). Five of six CR (complete response) + PR (partial response) samples and a majority of SD (stable disease) samples (13/19) had higher CTX-S scores (above the median; see Supplementary Fig. S4), supporting the CTX-S score as a reasonable measure of cetuximab disease control response. This notion was supported by CMH testing on cetuximab response versus CTX-S quartile scores [q4 (highest), q3, q2, and q1 (lowest; Supplementary Table S10B and S10C)]. Moreover, KM analysis found that higher scores were significantly associated with better PFS in patients, regardless of KRAS mutation status (log-rank test trend, P = 0.0026, n = 80; Fig. 1C) as well as in patients with only WT KRAS (P = 0.0320, n = 43; Fig. 1D).

Validation set 3 (in vitro). Recently, 147 colorectal cancer cell lines with heterogeneous genetic backgrounds were analyzed by Medico and colleagues (20) for KRAS, NRAS, and BRAF genotypes, MSI (MSI-high) status, as well as global gene expression, and in vitro CTX-S. Our further analysis of these cell lines showed that the CTX-S score was significantly correlated with in vitro CTX-S (Fig. 1E), supporting the validity of the CTX-S score.

The CTX-S score was not prognostic

We have previously developed various gene expression signatures that were prognostic for colorectal cancer outcomes (14, 26). To assess prognostic potential, a KM analysis of the CTX-S score by quartiles was performed similarly as we did for the ΔPC1.EMT score in the same set of 468 colorectal cancers (14). Results showed that unlike ΔPC1.EMT that predicted poor OS (14), the CTX-S score was not prognostic, as shown in either all patients (log-rank trend test, P = 0.969, n = 468), or in WT KRAS patients (P = 0.273, n = 264; Supplementary Fig. S5A and S5B). This result was confirmed using a second large, independent, set of stage I–IV colorectal cancers (n = 557) reported by Marisa and colleagues (2013) for a classification analysis of colorectal cancer in association with prognosis (relapse-free survival, RFS; ref. 22; Supplementary Fig. S6A and S6B).

Correlation of the CTX-S score with EREG/AREG, RAS, and Wnt signatures

Spearman correlation analysis showed that while the CTX-S score was positively correlated with EREG or AREG expression in all four patient tumor datasets (P < 0.0001), it was negatively correlated with 18-gene RAS signature score in the three largest datasets (P < 0.0001; Table 2A). Notably, an 18-gene RAS pathway gene expression signature score was previously developed to measure RAS/RAF/MEK pathway activation (27). We recently adapted this signature from use in fresh frozen colorectal cancer samples to more clinically available, formalin-fixed paraffin-embedded tissues (34) as a means to identify cetuximab nonresponders.

The Wnt pathway is another major dysregulated signaling pathway in colorectal cancer, approximately 75% of which have one or two APC truncating mutations (15, 21, 35). We found a strong correlation with an APC mutation–dependent 24-gene Wnt pathway score across all five datasets tested. In addition, we found that the APC mutation–dependent score was significantly correlated with the in vitro cetuximab-inhibitory effect in 147 cell lines (Supplementary Fig. S7).

These data led us to further investigate a potential association of the CTX-S score with APC mutations using Moffitt colorectal cancer patients. We found that CTX-S scores were significantly higher (P < 0.0001 for two-tailed Welch t test) in mutant APC (n = 312, 67%) than WT APC tumors (n = 156, 33%; Supplementary Fig. S8A). Similar results were obtained when tumors were further divided into WT and MUT RAS (KRAS/NRAS) or MSI and MSS (Supplementary Fig. S8B and S8C). It was also true when the effect of MUT APC was examined in Moffitt stage IV patients (n = 110; Supplementary Fig. S8D–S8F) and in TCGA colorectal cancers (Supplementary Fig. S9A).

Identification of APC and TP53 as the highest-ranked CTX-S–associated mutated genes

The finding of a potentially significant role of mutant APC in predicting CTX-S prompted us to examine additional genes. We applied an analytical approach fusing RNA-based gene expression signatures with DNA mutations to rank mutated genes in Moffitt colorectal cancers (see Materials and Methods). The top 20 ranked mutated genes are shown in Table 2B, and a full rank list of mutated genes is given in Supplementary Table S11. Although MSI status, BRAF ([i.e., BRAF (V600E)], and TGFBR2 were the most negatively correlated with the CTX-S score, TP53 and APC were the highest ranked mutated genes that were strongly (positively) correlated with the scores (Table 2B, left). Here, we treated the MSI status as “a mutated gene.” Notably, many negatively correlated genes, such as BRAF and TGFBR2, were strongly associated with MSI (15, 21, 35). After MSI tumors were removed, all the negatively correlated mutated genes had statistically nonsignificant, Padj values while TP53 and APC remained the only statistically significant positively correlated genes (Table 2B, right; Supplementary Table S12). This is supported by an analysis showing striking trends by multiple APC genotypes and TP53 mutations (Supplementary Table S13).

APC+TP53 doubly mutated (AP) tumors had the highest CTX-S scores

Because APC and TP53 were frequently comutated in colorectal cancer tumors (15, 21, 35), we examined whether mutant APC+TP53 together might cooperatively predict CTX-S. For this purpose, Moffitt colorectal cancers were divided into four subgroups: (i) MUT APC + MUT TP53 (AP); (ii) MUT APC + WT TP53 (A); (iii) WT APC + MUT TP53 (P); and (iv) WT APC + WT TP53 (WT AP). Analysis indicates that doubly mutated AP tumors had significantly higher CTX-S scores than all other three subgroups including the A and P mutant groups (P < 0.0001 for two-tailed Welch t test; Fig. 2A, left). Notably, A and P mutant groups were not significantly different from each other, but had significantly
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Table 2A. Spearman correlations with the CTX-S score

<table>
<thead>
<tr>
<th>Gene expression/Signature scores</th>
<th>Moffitt colorectal cancers (n = 458)*</th>
<th>TCGA colorectal cancers (n = 624)</th>
<th>Marisa et al. (2013) colorectal cancers (n = 566)</th>
<th>Khambata-Ford et al. (2007) colorectal cancers (n = 80)</th>
<th>Medico et al. (2015) colorectal cancer cell lines (n = 147)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EREG</td>
<td>0.562</td>
<td>-0.0001</td>
<td>0.592</td>
<td>-0.0001</td>
<td>0.655</td>
</tr>
<tr>
<td>AREG</td>
<td>0.463</td>
<td>0.0001</td>
<td>0.469</td>
<td>0.0001</td>
<td>0.542</td>
</tr>
<tr>
<td>18-gene RAS pathway score</td>
<td>-0.361</td>
<td>-0.0001</td>
<td>-0.209</td>
<td>-0.0001</td>
<td>-0.491</td>
</tr>
<tr>
<td>64-gene Wnt pathway score</td>
<td>0.069</td>
<td>0.140</td>
<td>0.023</td>
<td>0.013</td>
<td>0.536</td>
</tr>
<tr>
<td>24-gene Wnt pathway score</td>
<td>0.553</td>
<td>-0.0001</td>
<td>0.457</td>
<td>-0.0001</td>
<td>0.558</td>
</tr>
</tbody>
</table>

NOTE: The remaining P values are significant after adjustments for multicomparisons by the Holm-Bonferroni method and are highlighted in bold.

*Of Moffitt 468 colorectal cancers, 10 samples lacking appropriate gene probe values for some signature scores were excluded from correlation analysis.

Table 2B. Ranking of CTX-S score-associated mutated genes using Moffitt colorectal cancers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>P</th>
<th>N</th>
<th>PCT</th>
<th>Adj Pct</th>
<th>Score</th>
<th>Gene</th>
<th>Mutation</th>
<th>P</th>
<th>N</th>
<th>PCT</th>
<th>Adj Pct</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td></td>
<td>1.89E-24</td>
<td>277</td>
<td>59.2%</td>
<td>0</td>
<td>5.8E-22</td>
<td>TP53</td>
<td></td>
<td>4.64E-22</td>
<td>261</td>
<td>64.1%</td>
<td>0</td>
</tr>
<tr>
<td>MSI_high</td>
<td></td>
<td>6.74E-24</td>
<td>61</td>
<td>15.0%</td>
<td>0</td>
<td>2.06E-21</td>
<td>APC</td>
<td></td>
<td>1.93E-23</td>
<td>62</td>
<td>13.2%</td>
<td>0</td>
</tr>
<tr>
<td>TGFBR2</td>
<td></td>
<td>1.93E-23</td>
<td>62</td>
<td>15.2%</td>
<td>0</td>
<td>5.9E-21</td>
<td>BRAF</td>
<td></td>
<td>0.000709</td>
<td>18</td>
<td>4.4%</td>
<td>0</td>
</tr>
<tr>
<td>APC</td>
<td></td>
<td>7.96E-21</td>
<td>312</td>
<td>66.7%</td>
<td>0</td>
<td>2.44E-18</td>
<td>Kras</td>
<td></td>
<td>0.001858</td>
<td>177</td>
<td>43.5%</td>
<td>0</td>
</tr>
<tr>
<td>BRAF</td>
<td></td>
<td>5.67E-17</td>
<td>53</td>
<td>11.3%</td>
<td>0</td>
<td>1.74E-14</td>
<td>SMAD3</td>
<td></td>
<td>0.00204</td>
<td>14</td>
<td>3.4%</td>
<td>0</td>
</tr>
<tr>
<td>CELSR2</td>
<td></td>
<td>1.55E-06</td>
<td>72</td>
<td>15.4%</td>
<td>0</td>
<td>0.000478</td>
<td>BARD1</td>
<td></td>
<td>0.00188</td>
<td>7</td>
<td>1.7%</td>
<td>0</td>
</tr>
<tr>
<td>HBLBP</td>
<td></td>
<td>4.03E-06</td>
<td>23</td>
<td>4.9%</td>
<td>0</td>
<td>0.002344</td>
<td>CTNNB1</td>
<td></td>
<td>0.005312</td>
<td>13</td>
<td>3.2%</td>
<td>0</td>
</tr>
<tr>
<td>ITGB4</td>
<td></td>
<td>6.05E-06</td>
<td>45</td>
<td>9.6%</td>
<td>0</td>
<td>0.001952</td>
<td>BRCM1</td>
<td></td>
<td>0.005571</td>
<td>25</td>
<td>6.1%</td>
<td>0</td>
</tr>
<tr>
<td>PML</td>
<td></td>
<td>6.3E-06</td>
<td>22</td>
<td>4.7%</td>
<td>0</td>
<td>0.001926</td>
<td>IDH1</td>
<td></td>
<td>0.006563</td>
<td>5</td>
<td>1.2%</td>
<td>0</td>
</tr>
<tr>
<td>HSPA2</td>
<td></td>
<td>1.25E-05</td>
<td>14</td>
<td>3.0%</td>
<td>0</td>
<td>0.003749</td>
<td>RAD1B</td>
<td></td>
<td>0.006847</td>
<td>6</td>
<td>1.5%</td>
<td>0</td>
</tr>
<tr>
<td>MLL2</td>
<td></td>
<td>2.29E-05</td>
<td>84</td>
<td>17.9%</td>
<td>0</td>
<td>0.006995</td>
<td>SMAD2</td>
<td></td>
<td>0.006852</td>
<td>12</td>
<td>2.9%</td>
<td>0</td>
</tr>
<tr>
<td>MICAL1</td>
<td></td>
<td>2.36E-05</td>
<td>65</td>
<td>13.6%</td>
<td>0</td>
<td>0.007217</td>
<td>TLE7</td>
<td></td>
<td>0.01293</td>
<td>5</td>
<td>1.2%</td>
<td>0</td>
</tr>
<tr>
<td>PTP1R</td>
<td></td>
<td>2.58E-05</td>
<td>47</td>
<td>10.0%</td>
<td>0</td>
<td>0.007785</td>
<td>HECW1</td>
<td></td>
<td>0.012866</td>
<td>24</td>
<td>5.9%</td>
<td>0</td>
</tr>
<tr>
<td>MAP3K9</td>
<td></td>
<td>2.72E-05</td>
<td>21</td>
<td>4.5%</td>
<td>0</td>
<td>0.008312</td>
<td>TRIB3</td>
<td></td>
<td>0.013672</td>
<td>8</td>
<td>2.0%</td>
<td>0</td>
</tr>
<tr>
<td>ITK1</td>
<td></td>
<td>4.23E-05</td>
<td>34</td>
<td>7.3%</td>
<td>0</td>
<td>0.012936</td>
<td>NRR2</td>
<td></td>
<td>0.008995</td>
<td>15</td>
<td>3.7%</td>
<td>0</td>
</tr>
<tr>
<td>HDAC4</td>
<td></td>
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NOTE: P value from normal scores test for comparing CTX-S scores of mutated and WT tumors for the given gene. Adj Pct was calculated using the Hochberg and Benjamini method (29). Here, top 20 genes are listed according to their Adj Pct values. See a full rank list of 132 cancer-associated genes in Supplementary Tables S11 and S12. The statistically significant, positively associated mutated genes are highlighted in bold.

Abbreviations: APC, APC truncated mutation; Asso Dir, the directionality of the association (+/-); BRAF, BRAF (V600E); N, number (of mutated tumors); PCT, percentage (of mutated tumors).

*Patients with MSI-high status.

higher scores than WT AP tumors (P < 0.0001). The same pattern was observed in the 110 stage IV tumors (Fig. 2A, right). Similar results were obtained in Moffitt WT RAS tumors for all stages (n = 264), and for stage IV (n = 54) patients (Fig. 2B). The significant association was validated using the TCGA colorectal cancer dataset (Supplementary Fig. S9B).

APK* triply mutated tumors were associated with higher CTX-S scores than other RAS-mutated tumors.

We found that the presence of MUT AP also had a striking positive effect on the CTX-S scores of KRAS/NRAS-mutated (K/N)-tumors in Moffitt colorectal cancers. Note that because the great majority of RAS mutations in colorectal cancers are KRAS mutations (~40%) and the frequency of NRAS mutations is much lower (~5%), for simplicity, we used K* to represent both KRAS and NRAS mutations (K/N). Although WT RAS tumors (n = 264) had significantly higher CTX-S scores (P < 0.0001 for two-tailed Welch test) than MUT RAS tumors (n = 111) in which the mutant APK* subpopulation was excluded, the APK* triply mutated tumors (n = 91) had even higher scores (P < 0.0001; Fig. 2C, left).

When restricted to Moffitt stage IV tumors (n = 110), a striking difference remained for mutated K* tumors between the APK* and non-APK* subpopulations (Fig. 2C, right). In the TCGA colorectal cancers, the mutant APK* tumors and WT RAS tumors had no significant difference in CTX-S scores but both had significantly higher scores than other RAS-mutated tumors (Supplementary Fig. S9C). These data suggest that some APK* patients (heretofore not treated) may be sensitive to CTX treatment.

Comparison of the CTX-S scores among seven MSI and MSS subgroups

We next examined the association of APC + TP53 doubly mutated (AP) tumors with the MSI/MSS status. In the 468 Moffitt colorectal cancers, very strikingly, there were only two mutant
Figure 2.
The "MUT APC + MUT TP53" (AP) doubly mutated tumors had significantly higher CTX-S scores than other tumors with "MUT APC only" (A), "MUT TP53 only" (P) or WT APC + WT TP53 (WT AP) in Moffitt colorectal cancers. A, Comparison for all stage patients (n = 468) and stage IV patients (n = 110), respectively, regardless of RAS mutation status. B, Comparison for all stage WT RAS patients (n = 264) and stage IV WT RAS patients (n = 55), respectively. C, Comparison of the CTX-S scores was also performed between WT RAS, APK*, [MUT APC + MUT TP53 + MUT KRAS(or NRAS)], and other MUT RAS tumors without APK*. The CTX-S scores were normalized by the mean of 468 colorectal cancers, and bars represent mean ± SEM. P values for two-tailed Welch t test are shown. D, The CTX-S scores were compared between MSI (i.e., MSI-high) and MSS tumors (left) in all patients (n = 468). Bars, Median with interquartile range. The comparison was also made for the percentage of "MUT APC + MUT TP53" (AP), with "*" representing two-tailed P values for Barnard exact test (right; see Supplementary Table S14A). E, Comparison of CTX-S scores among 1 MSI and 6 MSS subgroups in Moffitt colorectal cancers (n = 468). Bars, Median with interquartile range. Unadjusted P values for two-tailed Welch t test are shown, and those remaining being significant after adjustments for multicomparisons by Holm–Bonferroni method are highlighted by maroon color.

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A Predictive Role for APC and TP53 Mutations
Figure 3.
Both the CTX-S score and the percentage of "MUT APCC + MUT TP53" (AP) tumors were significantly higher in left-sided tumors in Mofitt and TCGA colorectal cancers. A diagram of the CTX-S scores (high to low) versus 7 MUT/WT APCC/TP53/RAS(KRAS/NRAS)/BRAF subgroups in MSI and MSS tumors was displayed for Mofitt (n = 464; A) or TCGA (n = 217; B) datasets. Of 468 colorectal cancers, 4 samples without tumor location information were excluded; of 221 colorectal cancers, 3 samples without tumor location information and 1 sample with MSI status information were excluded. Comparison of the CTX-S scores between "Left" and "Right" colorectal cancers was made in all (available-data) patients, WT RAS patients, and WT RAS/RAF patients on Mofitt (C) and TCGA (D) colorectal cancers, respectively. WT RAS, patients with WT KRAS/NRAS; WT RAS/RAF, patients with WT KRAS/NRAS/BRAF; Bars represent median with interquartile range. P values are for two-tailed Welch t test. The comparison was also made for the percentage of "MUT APCC + MUT TP53" (AP; Left vs. Right). **P** values for Barnard exact test (see Supplementary Table S15 and S16). The AP tumors were predominantly CMS2 subtype. 

E, The plot of the number of tumors of 1 MSI and 6 MSS subgroups (similarly as defined in Fig. 2E) versus CMS1-4 and CMS_NA in Mofitt colorectal cancers (n = 458). Of 468 colorectal cancers, 10 samples without appropriate microarray data for the CMS classification analysis (16) were excluded as described previously (15). F, Left, Comparison of the percentage of "MUT APCC + MUT TP53" (AP; regardless of RAS mutation status) between CMS2 and other CMS subclasses in Mofitt MSS colorectal cancers (n = 399), with "**" representing a two-tailed P value of Barnard exact test. CMS_other includes CMS1, CMS3, CMS4, and CMS_NA (indeterminate). Right, Comparison of the CTX-S scores among CMS1-4 and CMS_NA subclasses in MSS colorectal cancers (n = 399). Bars, Median with interquartile range. P values are for two-tailed Welch t test. MUT, mutant.
AP tumors (1 APK and 1 APB) identified out of 61 MSI-H tumors (Fig. 3D). All 197 other AP tumors were MSS tumors. Similarly, the TCGA dataset had only one mutant AP tumor (APK) of 28 MSI cases (Supplementary Fig. S9D). The statistical significances of these associations are given using Barnard exact test (Supplementary Table S14).

Our data led us to postulate that AP mutations might cooperatively play a role in modulating CTX-S in MSS tumors. To more specifically examine this, we divided the Moffitt colorectal cancers into 1 MSI and 6 MSS subgroups. The groups can be statistically ordered with decreasing CTX-S scores as: AP > [APK, A, P] > AK+PK > K_MUT BRAF > WT AP, MSI, where the groups inside the brackets are not significantly different from each other (Fig. 2E). Similar results were also obtained in 220 TCGA colorectal cancers (Supplementary Fig. S10A) and in Moffitt stage IV patients (n = 110; Supplementary Fig. S10B). Taken together, these data suggest the clinically relevant, provocative possibility that some APK/RAS-mutant MSS tumors might benefit from EGFRi therapies.

Mutant AP genotype association with left-sided, MSS tumors explains biology and left-sided CTX sensitivity

We assessed whether the effect of mutant AP might be associated with the tumor sidedness. A plot of the CTX-S scores (high to low) of individual tumors with sidedness was produced to illustrate frequencies of the seven MSI and MSS subgroups [Left vs. Right; see Fig. 3A Moffit and (B) TCGA, respectively]. The CTX-S score was significantly higher in left-sided tumors than in right-sided tumors in all Moffit patients (n = 464, P < 0.0001, two-tailed Welch t test), WT tumors only (n = 262, P < 0.0001), and WT RAS/RAF patients (n = 209, P < 0.0001; Fig. 3C). Similar results were obtained for the TCGA dataset (Fig. 3D). The CMH test or Barnard exact test was performed on the frequencies of MSI and mutant AP by the sidedness (Left vs. Right) in Moffitt and TCGA colorectal cancers (Supplementary Tables S15 and S16). As expected, MSI was strongly associated with right-sided tumors (P < 0.0001) in both datasets. Moreover, in close association with significantly differing CTX-S scores, the percentage of AP tumors was significantly higher in left-sided than right-sided cases in both datasets (Fig. 3C and D). We further performed the distribution analysis and CMH tests of the CTX-S score by quartiles for different A/P/K/B subgroups [Left vs. Right] in the Moffitt 464 patients (see Supplementary Table S17A–S17E). The quartiles with higher scores (Q4 or Q3) are predicted to be more responsive to cetuximab. Briefly, this subset analysis shows while left-sided AP tumors have 92% (77/84) of cases with cetuximab scores greater than the median, 82% (14/17) of right-sided AP tumors also exceed the median. Moreover, left- and right-sided APK tumors have CTX-S scores greater than the median in 76% (37/49) and 73% (30/41) of cases, respectively. These data compare with all other tumors without AP mutations, where only 26% (72/276) of cases greater than the median, including only 41% (44/108) when restricted to KRAS/NRAS/BRAF WT tumors. Thus it appears that CTX-S is driven by APK + TP53 mutations that may overcome sidedness and/or KRAS/NRAS mutation status to some extent. Consequently, some right- and left-sided tumors not currently considered for therapy may benefit. In addition, analysis using the two-tailed Welch t test showed no significant left versus right CTX-S score difference in any particular subgroup in Moffitt dataset (Supplementary Fig. S11). These data suggest that the mutation frequencies, rather than the CTX-S scores, may be responsible for the sidedness effect.

Unlike the AP, single-driver mutations were not consistently associated with the sidedness

Single-driver mutations such as KRAS, NRAS, and BRAF mutations have been used in predicting EGFRi responses (5, 6, 8). We performed Barnard test to examine whether there is a potential association of the frequencies of four single drivers APC, TP53, KRAS/NRAS, and BRAF with the sidedness in the Moffitt dataset (Supplementary Table S18) and the TCGA dataset (Supplementary Table S19). We observed a few, but scattering, significant associations that (i) MUT APC was significantly more left-sided in TCGA among all patients (n = 217, P = 0.0002), but not for MSS patients (n = 190, P = 0.23) and (ii) the same is also true for MUT TP53; (iii) MUT KRAS/NRAS was only significantly associated with right-sided tumors in Moffit MSS patients; (iv) As expected, MUT BRAF (that was strongly associated with MSI tumors) was significantly associated with right-sided tumors in both Moffitt and TCGA all patients. However, when MSI cases were removed, the association of BRAF mutations became insignificant in MSS patients of both datasets, with very low counts of BRAF-mutated tumors.

APK patients were more often distant metastatic, whereas WT AP MSS tumors were associated with mucinous histotypes

We also examined the distribution of age, stages, and histotypes as well as other clinicopathologic parameters in the MSI and 6 MSS subgroups of Moffitt colorectal cancers (see Supplementary Table S20). MSI was significantly associated with stage I–II (60%, P < 0.01, individual χ² contribution) and less associated with stage IV (10%, P < 0.05) and distant metastasis (11%, P < 0.01). In contrast, the APK patients were more often stage III (45%, P < 0.05) and distantly metastatic (49%, P < 0.05). Interestingly, the WT AP subgroup (having low CTX-S scores) was significantly more associated with the mucinous histotype (32%, P < 0.001), whereas the "cetuximab-sensitive" AP tumors appeared to be least mucinous (3%, P < 0.05).

AP mutations were predominant in the CMS2 subtype linked to Wnt and MYC activity

The CMS were recently created from a comprehensive molecular analysis of thousands (n = 4,151) of human tumors to best define colorectal cancer (16). We performed CMS classification in Moffitt colorectal cancers (see Supplementary Methods and Table S21). Results show that the majority of AP MSS tumors (57%) were the CMS2, whereas APK tumors were also more associated with the CMS2 subtype than all other CMS classes, whereas most of the 59 MSI tumors were the CMS1 subtype (Fig. 3E). The strong association of the AP/APK MSS tumors with the CMS2 was confirmed by Barnard exact test (Fig. 3E, left). Furthermore, we found that the CTX-S scores of the CMS2 type were significantly higher (P < 0.0001) than all other CMS classes (Fig. 3E, right).

AP mutations associated with better outcomes in PDX models treated with cetuximab

To test our hypothesis, we identified two published cetuximab-treated colorectal cancer patient-derived tumor xenograft (PDX) datasets [Julien and colleagues, n = 52 PDX models (23); Bertotti...
and colleagues (n = 98 PDX models (24)), which also had APC and TP53 mutation data (see Supplementary Tables S7 and S8). We performed the CMH trend test on cetuximab response by frequencies of A and/or P mutations in these two datasets. (i) For the Julien dataset, when the frequencies of AP mutations versus A or P mutations versus WT AP were compared between CR/PR/SD versus PD tumors, the CMH trend value was 0.0277 in 52 all PDX models or 0.0592 (marginally significant) in 25 WT RAS models (Table 3). (ii) For the larger Bertotti dataset, however, when compared between PR & SD versus PD tumors, the CMH trend value was 0.0008 in 98 PDX models that had WT KRAS, NRRAS, BRAF, and PIK3CA (Table 3). In addition, the CMH test was also performed when PR (n = 23) and SD (n = 50) cases were separated, with a P value of 0.0133 (Supplementary Table S22). Notably, the frequency of AP mutations had no difference between PR and SD cases (83% vs. 84%). These data again support a cooperative role of AP mutations in positively predicting cetuximab response (CR/PR and SD).

## Discussion

We have developed and validated a CTX-S signature score, using outcomes data from two independent, prospective clinical trials, as well as by an in vitro cetuximab-treated cell line dataset. The robustness of the CTX-S score is also supported by the findings that: (i) the score was not prognostic; (ii) the score had a strong correlation with EREG and AREG, the predictive biomarkers of cetuximab response (4, 6, 36, 37); (iii) the score was significantly associated with the CMS2 subtypes, which were recently reported to be associated with better cetuximab response (38, 39).

Because of the limited availability of clinical trial tissue samples with cetuximab exposure (especially mutant RAS patients), clinical outcomes, and deep molecular analysis beyond RAS/RAF testing, we used the CTX-S score as a "proxy" for clinical response. Using an integrated analytic approach we described previously (40), with the "lens" of gene expression, we identified a 2-gene mutation signature (APC+TP53; AP) to predict CTX-S, which was subsequently validated using two independent PDX datasets.

Identification of the 2-gene mutation signature (AP) has also provided new molecular insight into the recent observation that patients with MSI (often right-sided) almost uniformly lack AP mutations, and thus are resistant to EGFRi treatment, whereas patients with left-sided tumors more commonly harbor AP mutations, and are thus more responsive (6, 12, 13, 41, 42). Notably, it is the combination of AP mutations, rather than single-driver mutations (APC, TP53, KRAS/NRRAS, or BRAF), that was consistently and significantly associated with the sidedness. Currently, only left-sided colorectal cancers with a WT KRAS/NRRAS/BRAF status are considered eligible for EGFRi therapy (6). However, our further analysis suggests that mutations, rather than sidedness, may ultimately determine sensitivity to cetuximab. This is supported by the distribution analysis of the CTX-S score by quartiles for Left versus Right (10%–60%) of mutant APR/K/A/P/K, respectively (Table 3). In either-sided tumors, an AKP was the more "sensitive" subgroups, whereas WT AP (that also had WT RAS/RAF and often had mucinous histotypes) was one of the highly "resistant" subgroups.

These data have two important clinical implications that may facilitate achieving the goal of precision cancer care. First, they redefine the current clinical strategy guided by extended RAS/RAF testing and sidedness by excluding currently eligible, left-sided WT AP tumors, but including currently ineligible, right-sided MUT AP tumors. Second, the unexpected finding of a strong association of AKP with predicted CTX sensitivity suggests a potential therapeutic opportunity, requiring further clinical validation, to a subgroup of previously excluded RAS patients with a poor prognosis who harbor AKP mutations (15). For example, a recent analysis of gene expression markers on CALGB 80203 (Alliance) trial data reported that high expression of CD73 was associated with longer PFS from cetuximab both in WT KRAS patients (chemo + cetuximab: HR = 0.91 vs. chemo only: HR = 1.57; interaction = 0.026) as well as in mutant KRAS patients (chemo + cetuximab: HR = 0.80 vs. chemo only: HR = 1.29; interaction = 0.025; ref. 43), suggesting an intriguing and provocative hypothesis that some fraction of mutant KRAS patients may actually benefit from EGFRi therapies. Moreover, a number of clinical trial analyses have indicated that a substantial percentage (10%–60%) of mutant KRAS patients treated with cetuximab/panitumumab have achieved stable disease (SD, refs. 4, 36, 37).
44–51). Furthermore, analysis of the FIRE-3 (AIO KRK-0306) study showed that for the FOLFIRI + cetuximab treatment, compared with the WT KRAS exon 2 patients ([n = 297] who had 13 (4.4%) CR, 147 (57.6%) PR, and 52 (17.5%) SD), RAS-mutant patients ([n = 97]) achieved 1 (1.0%) CR, 36 (37.1%) PR and significantly higher SD (31, 32.0%; ref. 51). Finally, preclinical studies have indicated that EGFRi resistance can be reversed in some mutant KRAS colorectal cancer cell lines (52), and some KRAS-mutated colorectal cancer tumors may be decoupled from RAS pathway activation (25).

Our data suggest a hypothesis by which mutations in APC+TP53 might enable Wnt and p53 pathway “cross-talk” to transactivate the EGFR pathway, essentially addiciting tumors to EGF ligands or enhance antibody-dependent cellular cytotoxicity. Activation of the EGFR–PI3K–AKT signaling pathway has been clearly demonstrated in the APC–/– mouse by a mechanism involving upregulation of PGE2 (53, 54). Similar to WNT, the p53 pathway has cross-talk with the EGFR pathway. Specifically, mutant TP53 has been shown to induce ERG1 transcription that is driven by p-ERK (55, 56). We found that the APC+TP53 double-mutated tumors were predominantly the CMS2 subtype that was associated with WNT and MYC activation and frequent mutations in either APC or TP53 (16).

In conclusion, we have identified a 2-gene signature in identifying cetuximab-sensitive subpopulations. The signature may be useful in refining the appropriate subpopulation of patients for EGFRi treatment. Moreover, our findings provide a rationale for further prospective clinical studies that add sequencing of APC+TP53 to extended RAS/RAF testing to improve and expand the clinical utility of EGFRi—even potentially to some previously excluded patients harboring mutant RAS.

Disclosures of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Yang, M.J. Schell, A. Loboda, M. Nebozhyn, J. Li, J.K. Treer, W.J. Pledger, T.J. Yeatman
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References
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