Scarcity of Recurrent Regulatory Driver Mutations in Colorectal Cancer Revealed by Targeted Deep Sequencing

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Abstract

Background: Genetic testing of cancer samples primarily focuses on protein-coding regions, despite most mutations arising in noncoding DNA. Noncoding mutations can be pathogenic if they disrupt gene regulation, but the benefits of assessing promoter mutations in driver genes by panel testing has not yet been established. This is especially the case in colorectal cancer, for which few putative driver variants at regulatory elements have been reported.

Methods: We designed a unique target capture sequencing panel of 39 colorectal cancer driver genes and their promoters, together with more than 35 megabases of regulatory elements focusing on gene promoters. Using this panel, we sequenced 95 colorectal cancer and matched normal samples at high depth, averaging 170× and 82× coverage, respectively.

Results: Our target capture sequencing design enabled improved coverage and variant detection across captured regions. We found cases with hereditary defects in mismatch and base excision repair due to deleterious germline coding variants, and we identified mutational spectra consistent with these repair deficiencies. Focusing on gene promoters and other regulatory regions, we found little evidence for base or region-specific recurrence of functional somatic mutations. Promoter elements, including TERT, harbored few mutations, with none showing strong functional evidence. Recurrent regulatory mutations were rare in our sequenced regions in colorectal cancer, though we highlight some candidate mutations for future functional studies.

Conclusions: Our study supports recent findings that regulatory driver mutations are rare in many cancer types and suggests that the inclusion of promoter regions into cancer panel testing is currently likely to have limited clinical utility in colorectal cancer.
Detection of driver mutations in colorectal cancer has been shown to be an effective means of enabling therapy tailored to an individual patient. For example, colorectal cancer patients with RAS mutations respond poorly to anti–epidermal growth factor receptor cetuximab and panitumumab, and screening of patients for RAS mutations is now a routine aid in the decision of whether to administer anti–epidermal growth factor receptor therapy (7). Currently, targeted sequencing of specific cancer driver genes is typically achieved either by target enrichment through capture probes or by amplicon sequencing. Thus far, these methods have generally been designed to examine protein-coding regions. Determining whether mutations exist in regulatory elements of cancer driver genes has not been examined in colorectal cancer beyond the use of whole-genome sequencing (WGS) datasets, which are of relatively low coverage (40×) (8). Recently, sequencing data from an assay capturing regulatory elements in addition to protein-coding regions in a large cohort of breast cancers led to the identification of recurrent somatic mutations in the promoter of the cancer driver gene FOXA1 (5). The results of this study suggest that a targeted sequencing approach that includes regulatory regions may be useful for identifying regulatory driver mutations in other cancer types.

In our study, we performed target capture sequencing (TCS) to generate high-depth sequencing data across the promoter elements and coding regions of 39 colorectal cancer driver genes from 95 colorectal cancer and matched normal samples. In addition, we incorporated more than 35 megabases of selected regulatory regions, focusing on the promoters of all coding genes, and we comprehensively assessed somatic mutations in regulatory elements in search of noncoding driver mutations.

Methods

Patient Samples

A total of 95 colorectal cancer and matched normal samples were selected from a biobank of fresh tumor tissue and blood collected with patient informed consent. The study was carried out with ethics approval from the South Eastern Sydney Local Health District Human Research Ethics Committee (approval number H00/022 and 00113), and all samples were collected with written informed consent from each subject. Patient and sample characteristics can be found in Supplementary Tables 1 and 2 (available online).

TCS Assay Design and Analysis of Sequencing Data

A unique TCS assay encompassing 35,726,928 nucleotides of the genome was designed to provide sequencing data covering promoters, other regulatory regions, and some coding exons. WGS was also performed on one sample (see Supplementary text, available online). See Mendeley data for description and list of regions and details of analysis.

Variant Detection and Analysis

Somatic single-nucleotide variants were detected with Strelka (9). We defined high-confidence somatic mutations as those with variant allele frequencies 8.5% or higher (see Supplementary Figure 1A, available online) because this threshold led to sample-specific TCS mutation loads that were similar to those of other colorectal cancers (10), indicating mutations that are more likely to be true positives. Somatic indels were detected using SvABA (11) and Lancet (12) (see Supplementary text [available online] for detailed description of analysis).

Mutational Spectrum Analyses

Mutational spectra were assessed through Pearson correlation of trinucleotide frequencies in a given sample with mutational signatures (13) from the Catalogue of Somatic Mutations in Cancer (COSMIC) (14) database. Mutational spectra from TCS were normalized using genome trinucleotide frequencies. All Pearson correlations (r) reported had P less than .0001, indicating a correlation coefficient that is statistically significantly different from zero.

Experimental Validation of Variants Detected

Some somatic mutations were randomly selected for experimental validation via Sanger sequencing of polymerase chain reaction product amplified from cancer and matched normal patient DNA. Indels in the putative promoter of MTERFD3 were also validated using the same method.

Data Availability

TCS and WGS data from this study are available upon application via the European Genome-Phenome Archive with accession number EGAD00001004582. Mendeley data associated with this study can be downloaded from dx.doi.org/10.17632/c65rsd4fr2.1.

Results

TCS and Mutation Detection

We designed a TCS assay encompassing 35,726,928 nucleotides of the genome (Figure 1A). The assay was designed to focus on the promoters and exons of a panel of known colorectal cancer-associated genes (n = 39 genes; see Mendeley data). The assay also incorporated promoter regions of all known coding genes (n = 26,455 regions) and selected regulatory elements, including DNase I hypersensitivity sites (n = 13,891 regions), long noncoding RNAs (n = 842 regions), and microRNAs (n = 25 regions). With this unique TCS assay, we sequenced 95 colorectal cancer and matched normal samples (Supplementary Tables 1 and 2, available online). Average (standard deviation [SD]) reads per sequenced base were 169.96 (25.08) (SD calculated across 95 samples) in the cancer samples and 81.81 (17.13) in the matched normal samples. (Figure 1B). The percentage on-target rate across TCS cancer samples was 82.33 (1.72)% (see Mendeley data).

We identified 43,915 somatic single-nucleotide mutations across our cohort, with a median of 178 mutations per sample (see Methods; Supplementary Table 2, available online) and identified 5,244 somatic indels across our cohort, with a median of seven indels per sample (see Methods; Supplementary Figure 1B, Table 2, available online). We validated a selection of point mutations and a small deletion via Sanger sequencing (see Methods; Supplementary Figure 1, C and D, available online). To ensure that our TCS assay was able to capture somatic mutations in regulatory regions, we additionally performed WGS on sample
Figure 1. Sequence coverage by target capture sequencing (TCS) and mutation characteristics. A) Types and sizes of regions sequenced by TCS. B) Average per sample read coverage across sequenced bases in cancer and matched normal TCS samples. Read coverage is plotted for each region type. Dotted lines indicate average read coverage in cancer and matched normal samples across the cohort. C) Somatic single-nucleotide mutation rate per megabase (Mb) of each sample in the TCS cohort (n = 95), plotted on a log scale (y-axis). Colors represent colorectal cancer subtypes as indicated, and somatic single-nucleotide and indel mutations in colorectal cancer driver genes of interest are marked by bars. POLE Exonuc = Polymerase Epsilon exonuclease domain mutation; trunc = frameshift or stop-gain mutation; ns = nonsynonymous mutation. D) Numbers of indels identified in microsatellite unstable (MSI) and microsatellite stable (MSS) colorectal cancer samples sequenced by TCS. Individual samples are indicated by dots corresponding to the number of indels identified by at least two variant detectors. E) Average somatic single-nucleotide mutation rate (mutations per Mb) in cancer samples across region types sequenced by TCS. Data from somatic single-nucleotide mutations in The Cancer Genome Atlas (TCGA) whole-exome sequenced (WXS) colorectal cancer samples are shown, as indicated on the rightmost bars on the graph. WXS samples harboring nonsynonymous POLE coding variants are excluded, and coding exons are here derived from GENCODE (35) v29 data. F) Average variant allele frequency (VAF) of somatic single-nucleotide mutations overlapping exons of sequenced driver genes and VAF of all other mutations. All data represented, bar plots, error bars, and mid-line indicate mean and standard deviation, and ****P < .0001.
CRC_1, which had the highest mutation load. Over the regions captured by TCS, we found that the TCS assay identified 94.5% (n = 4585 of 4854) of mutations detected by WGS, suggesting that the assay is robust (for detailed comparison, see Supplementary text and Supplementary Figures 2–5, available online).

Characterization of Cohort According to Mutation Profiles

Mutation loads across our cohort were mostly consistent with previous observations among colorectal cancers (10,15) (Figure 1C), with generally increasing numbers of mutations across samples that were microsatellite stable (MSS; n = 80) and then microsatellite unstable (MSI; n = 15). We found that three MSS samples with high mutation loads harbored POLE exonuclease domain mutations (CRC_1: p.Pro286Arg; CRC_2: p.Met444Lys; CRC_8: p.Ser297Phe), which is known to result in an ultramutator phenotype (see Supplementary text, available online, for description of MSI and POLE mutation status annotation). As expected, MSI samples harbored statistically significantly more indels compared with MSS samples (Figure 1D). MSI samples in our cohort more commonly harbored BRAF V600E mutations (MSI: 8 of 15; P < .0001, Fisher’s exact test; Figure 1C) and RNF43 mutations (MSI: 7 of 15; P < .0001, Fisher’s exact test), and less commonly harbored APC variants (MSI: 5 of 15; P = .0044, Fisher exact test), than did the POLE exonuclease domain wild-type MSS samples (BRAF V600E mutation: 4 of 77; RNF43 mutation: 1 of 77; APC mutation: 58 of 77). We found recurrent mutations in genes such as SMAD4 (n = 10/95), ARID1A (n = 8/95), and SOX9 (n = 8/95) (Table 1), which have also previously been reported in colorectal cancer (10). Mutation loads and variant allele frequencies of somatic single-nucleotide mutations across different types of genomic regions captured in our TCS assay are shown in Figure 1, E and F, respectively.

Association of Deleterious Germline Mutations With Mutational Signatures

We next analyzed germline variants in our samples, finding three patients with putative pathogenic germline variants using ClinVar (16) annotations (see Methods and Mendeley data). These variants are nonsynonymous and truncating heterozygous variants in regions coding for MSH6, MUTYH, and ATM. The two samples that harbored a variant in either MSH6 or MUTYH showed distinct mutational spectra based on our TCS assay, and we examined these variants further.

Sample CRC_4 harbored a germline heterozygous C>T single-nucleotide polymorphism at chr2: 48 030 588 (Supplementary Figure 6A, available online; see Mendeley data), resulting in the introduction of an early stop codon at p.Arg1068Ter, a variant recorded in the InSIGHT database (17) as Class 5 pathogenic. A potential somatic second hit is a truncating G>A mutation in MSH6 at chr2: 48 026 216 (p.Trp365Ter). Sample CRC_4 was the fourth-most highly mutated sample in our cohort, and we found indel mutation numbers in this sample to be elevated when compared with MSS samples. The mutational spectrum in CRC_4 was strongly correlated with mismatch repair deficiency-associated signatures 14 and 6 (13, 18) from the COSMIC (14) database (r = 0.784 and r = 0.767, respectively, with P < .0001 by Pearson correlation; signature 14 shown in Figure 2A). Together with the relatively early age of colorectal cancer diagnosis in this patient (51 years, presenting with synchronous cancers of the rectum and sigmoid) and the microsatellite instability we observed in sample CRC_4, these findings are consistent with Lynch syndrome.

Our analysis of germline variants also led to the detection of one sample with a heterozygous germline C>T variant in the MUTYH gene at chr1: 45 798 117 (Supplementary Figure 6B; see Mendeley data). This variant has an allele frequency of 1.339 × 10^-4 in the Exome Aggregation Consortium database (19), and it results in a nonsynonymous amino acid change in MUTYH (p.Arg242His). The variant has been shown in vitro to severely impair glycosylase and DNA binding activity (20). The mutation burden of MSS sample CRC_3 is unusual (n = 2767 mutations) because it is higher than all MSI samples in our cohort. We did not observe a putative second somatic hit in MUTYH, and the region did not exhibit signs of loss of heterozygosity. However, interestingly the mutational spectrum of this sample was highly correlated with the COSMIC (14) database’s signature 18 (r = 0.825 and P < .0001 by Pearson correlation; Figure 2B), and this signature has been associated with defects in the base excision repair pathway and MUTYH deficiency (21).

Mutations in the Promoters of Cancer Driver Genes

Having shown that our TCS method accurately captured mutational spectra and both germline and somatic variants, we then examined mutations in the promoters of our set of 39 driver genes to search for putative recurrent regulatory driver mutations. We identified 33 somatic single-nucleotide and indel mutations in the promoters of these genes (see Mendeley data). Of the 39 promoters, TCF7L2, VTT1A, SOX9, BMP3, APC, RSP03, and BRAF were mutated in two or more samples. To assess the potential impact of these mutations, we sought to identify a second hit to each of these genes in the same sample, such as a deleterious coding mutation or a loss of heterozygosity event (where this was possible to determine using heterozygous

### Table 1. Summary of driver mutations in genes sequenced, with more than 5% recurrence in the sequenced cohort

| Gene     | MSS (n = 77) | MSI (n = 15) | POLE mut. (n = 3) | Total (n = 95) |
|----------|-------------|-------------|------------------|---------------|
| APC      | 58 (75.3%)  | 5 (33.3%)   | 3 (100.0%)       | 66 (69.5%)    |
| TP53     | 53 (68.8%)  | 2 (13.3%)   | 2 (66.7%)        | 57 (60.0%)    |
| KRAS     | 22 (28.6%)  | 2 (13.3%)   | 1 (33.3%)        | 25 (26.3%)    |
| FBXW7    | 11 (14.3%)  | 4 (26.7%)   | 2 (66.7%)        | 17 (17.9%)    |
| PIK3CA   | 10 (13.0%)  | 2 (13.3%)   | 3 (100.0%)       | 15 (15.8%)    |
| BRAF     | 6 (7.8%)    | 8 (53.3%)   | 0 (0.0%)         | 14 (14.7%)    |
| TCF7L2   | 7 (9.1%)    | 1 (6.7%)    | 2 (66.7%)        | 10 (10.5%)    |
| SMAD4    | 8 (10.4%)   | 2 (13.3%)   | 0 (0.0%)         | 10 (10.5%)    |
| RNF43    | 1 (1.3%)    | 7 (46.7%)   | 1 (33.3%)        | 9 (9.5%)      |
| SOX9     | 7 (9.1%)    | 1 (6.7%)    | 0 (0.0%)         | 8 (8.4%)      |
| ARID1A   | 2 (2.6%)    | 4 (26.7%)   | 2 (66.7%)        | 8 (8.4%)      |
| PTPRK    | 2 (2.6%)    | 4 (26.7%)   | 1 (33.3%)        | 7 (7.4%)      |
| PMS1     | 3 (3.9%)    | 1 (6.7%)    | 1 (33.3%)        | 5 (5.3%)      |
| MSH6     | 1 (1.3%)    | 2 (13.3%)   | 2 (66.7%)        | 5 (5.3%)      |

*The number of samples with at least one somatic single-nucleotide or indel mutation in each gene is shown. For a mutation to be considered a driver, it must be either a frameshift or stop-gain mutation, or a missense mutation with a PROVEAN (37) converted rankscore of more than 0.5, a PolyPhen (38) prediction of “deleterious,” or a SIFT (39) prediction of “possibly damaging” or “probably damaging.” The percentage listed indicates the fraction of the subtype or total cohort that harbored at least one mutation fulfilling these criteria. MSI = microsatellite unstable sample; MSS = microsatellite stable sample; POLE mut. = Polymerase epsilon exonuclease domain-mutated MSS sample.

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We also captured the promoter of the TERT gene in our TCS assay, a gene that harbors the most frequent regulatory driver mutations yet discovered in cancer (22,23). We investigated whether samples in our cohort also harbored somatic mutations at chr5: 1, 295, 228 and chr5: 1, 295, 250 within the TERT promoter, but we found that none of our cancer samples carried these mutations (see Mendeley data). The TERT promoter was also devoid of any somatic single-nucleotide or indel mutations, confirming previous reports that such mutations are of low frequency in colorectal cancer (24,25).

Search for Other Potentially Functional Mutations in Regulatory Regions

We next utilized our full mutation dataset covering all captured regions in search of other potentially functional regulatory mutations. We used OncodriveFML to search for functional enrichment in genomic regions (26). We did not find any functional enrichment of mutations in our cohort beyond coding exons of the driver genes sequenced (Figure 3C). Assigning function to a noncoding variant can be imprecise because of the variety of ways in which a variant may affect gene regulation (1), so we also considered base pair recurrence of somatic variants within our cohort. To improve filtering of variants in our TCS cohort (n = 95 samples), we also incorporated single-nucleotide variants from WGS colorectal cancer samples from TCGA (n = 60; see Mendeley data). Within regulatory regions, we found 90 recurrent somatic single-nucleotide variants that were present in more than three samples, with at least one sample from each of the TCS and TCGA cohorts (see Mendeley data). These variants were then prioritized by FunSeq2 (27), which selected 47 variants (see Mendeley data) as candidate functional mutations by designating a high noncoding variant score or an association with any cancer gene. These mutations were in proximity to cancer-related genes such as JUN, CDKN1B, and ASP1A. Further investigating these 47 mutations identified as candidates by FunSeq2, we selected any mutations that were present in four or more TCGA colorectal cancers and examined expression of nearby gene(s) in wild-type and mutant samples (n = 4 mutations selected). We investigated the expression of any gene within 6 kilobases (kb) of the variant but found no statistically significant difference in gene expression via RNA-sequencing by unpaired t test between TCGA samples that were wild-type and mutant for each variant.

Finally, we investigated recurrent indel mutations that lay outside of the coding exons of the driver genes sequenced. Counting indels in our TCS cohort that arose within our sequenced regions in defined genomic windows (indel locus ± 10 base pairs [bp]), we identified 16 candidate windows (21–28 bp) harboring a total of 66 indels (see Methods and Mendeley data). Windows were selected as candidates if they harbored at least four indels, or three or more indels if at least one sample was MSS. With the exception of the recurrent indels within the VT1IA promoter discussed previously, the noncoding indel with the highest transcription factor-occupancy lay within the region chr12: 107, 380, 956–107, 380, 983 (indels overlapping a maximum of 46 transcription factor chromatin immunoprecipitation sequencing annotations; n = 3 indels). The region lies within a putative promoter for the mitochondrial transcription termination factor (mTERF) mTERFD3 (Figure 3D), and we validated all three indels via Sanger sequencing (Figure 4A). The indels in our cohort overlap Factorbook (28) binding sites for transcription factors SP1/SP2, E2F4/E2F6, and MAZ (Figure 4B). Overexpression
of MTERFD3 and other mTERF family proteins has been associated with mitochondrial DNA copy number depletion (29), and mitochondrial DNA copy number variation can occur in cancer tissues (30). However, experimental functional validation would be required to determine whether these variants might contribute toward oncogenesis through such a capacity.
Discussion

In recent years, many recurrent mutations have been found within cis-regulatory regions of cancer genomes, but few drivers have yet been identified. We undertook this study to detect regulatory driver mutations in colorectal cancer and to determine whether it may be beneficial to include the sequencing of gene promoters into current somatic mutation testing panels in colorectal cancer. Our results suggest there are no strong candidate recurrent regulatory driver mutations in the promoters of key colorectal cancer driver genes and other regulatory regions that we sequenced. Our findings suggest such mutations may be rare in colorectal cancer more generally. Because of the current sparsity of recurrent regulatory driver mutation discoveries in colorectal cancer, it is unlikely to be beneficial at this stage to include the sequencing of gene promoters in current panel testing assays.

In this study, we list single-nucleotide variants and genomic windows containing recurrent indels that may be functional noncoding mutations. We selected these variants by measuring recurrence, FunSeq2 score (27), and annotations of transcription factor binding. There are a plethora of ways in which regulatory mutations may affect genome function. For example, a mutation may alter a transcription factor binding site, affect the partitioning of the genome into topologically associating domains, or cause epigenetic changes by altering the binding of pioneer factors.
factors, nucleosome positioning, chromatin organization, or CpG methylation (1). We have ultimately based our functional annotations on computational prediction methods, and it is possible that our computational approaches may have identified additional regulatory features. Our approach was also potentially applicable to noncoding driver detection in melanoma and blood cancers, for which a number of somatic regulatory driver mutations have already been well established (23,31–34).

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**Notes**

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