

## APC, FBXW7, KRAS, PIK3CA, and TP53 Gene Mutations in Human Colorectal Cancer Tumors Frequently Detected by Next-Generation DNA Sequencing

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### Abstract

Colorectal cancer (CRC) is the fourth leading cause of cancer deaths worldwide. Genetic mutations have been linked to 5-10% of CRCs, with other environmental, genetic, and epigenetic factors influencing development and progression of the cancer. As individual cancers exhibit unique mutation patterns, identification of the distinctive CRC profile is essential to develop more effective target therapies. In this study, we used Ion Torrent Ampliseq Cancer Panel to sequence 737 loci from 45 cancer-related genes to identify genetic mutations in 93 human colorectal cancer samples. Sequencing analysis revealed frequent missense mutations in APC (17.2%), FBXW7 (10.8%), KRAS (50.5%), PIK3CA (10.8%), and TP53 (23.7%) in CRC samples of various histologic types, and additional mutations were also detected in other genes (BRAF, CTNNB1, NRAS, and SMAD4) at lesser frequencies. We also found common combination mutations between KRAS and TP53 (12.9%), KRAS and APC (8.6%), and KRAS and PIK3CA (8.6%). Sequencing individual human cancers may be the key to developing more effective drugs to target individual, cancer-specific mutations. Identifying the complete mutation profile in CRCs for the application of personalized and tailored targeted therapy is critical for developing new cancer treatments. We believe a faster and cost effective genotyping tool such as Ion Torrent sequencing technology will be greatly beneficial for the assignment of such specific therapeutics for CRCs in the near future.

**Keywords:** Colorectal cancer; Genetic mutations; Ion torrent sequencing; Targeted therapy; Personalized medicine

### Introduction

Colorectal cancer (CRC) is the second most common malignancy in women and the third most common in men worldwide. In 2012, nearly 1.4 million new CRC cases were reported and roughly 694,000 associated deaths occurred worldwide, making it the fourth most common cause of death from cancer [1]. The same year, China alone accounted for more than 253,000 cases and nearly 140,000 CRC deaths. Nearly 60% of all CRC cases are reported from developed regions, and men have a significantly higher incidence and mortality rate than women [2]. While roughly one third of individuals diagnosed with CRC will die from the disease [3], an estimated 60% of CRC deaths could be prevented with routine screening of individuals over the age of 50 [4]. Despite continuing efforts to improve treatment options for colorectal cancer patients, the staggering incidence and high mortality rates prevail.

Like other cancers, a variety of environmental and genetic interactions are involved in the development of CRCs. Some major

etiologic agents which influence sporadic CRC include inflammatory bowel disease (IBD), old age, male gender, high fat diet and obesity, tobacco and alcohol consumption, and sedentary lifestyle, while inherited genetics are responsible for between 5 and 10% of CRC cases [5-8]. Some well characterized familial genetic syndromes have previously been identified which confer a higher risk of developing CRC: hereditary non-polyposis colorectal cancer (HNPCC), which is associated with mutated genes in the DNA mismatch repair pathway, including MSH2 and MLH1 [9,10]; and familial adenomatous polyposis (FAP), which is the consequence of germline mutations in the tumor suppressor gene APC. These APC mutations result in a non-functional APC protein product that fails to regulate the cellular overgrowth of polyps. Carriers of germline APC mutations have virtually 100% chance of developing CRC by age 40, and most sporadic cancers have somatic mutations or deletions in this gene [11]. Loss of APC function is often followed by a series of other tumor suppressor gene mutations, including KRAS, PIK3CA, and TP53 [12]. In addition, epigenetic modifications like DNA methylation, histone modifications, and non-coding RNAs are particularly common in CRCs compared to other cancers [13]. The complex heterogeneity of CRCs highlight the need to identify biomarkers useful for target

therapies, as these genetic and epigenetic alterations have been demonstrated to correlate with prognosis and treatment responses [10]. For example, CRC tumors with KRAS mutations do not respond to commonly used drugs targeting EGFR, and chromosome instability found in more than 80% of CRCs is often associated with multi-drug resistance [10,14].

Despite ongoing efforts to improve screening and treatment of colorectal cancers, further research is necessary to determine additional unknown genetic mutations and epigenetic influences which are involved in the development and progression of the disease. A variety of complex interactions between genetic and environmental factors contribute to the unique gene mutation profile exhibited in each individual tumor, and profiling an individual's cancer genome can reveal the oncogenic mechanisms that regulate the cancer. As such, there is accumulating evidence suggesting that individualized, tailored therapies are needed to more effectively treat cancer patients. Until recently, personalized medicine based on individual genome sequencing was expensive, time inefficient, and generally impractical; however, the recent development of semiconductor-based technology known as Ion Torrent sequencing, which differs from other sequencing methods in that it does not rely on modified nucleotides or optics, is making individual genome sequencing a possibility [15]. In this study, we have used Ion Torrent sequencing to analyze 93 clinical colorectal cancer samples to identify the genetic mutations in 737 loci from 45 known cancer-related genes.

## Materials and Methods

### Ethics statement

The study has been approved by the Human Research Ethics Committee of the First Affiliated Hospital of Dalian Medical University, China. The institutional ethics committee waived the need for consent for formalin-fixed, paraffin embedded (FFPE) tumor samples from the tumor tissue bank at the Department of Pathology of the hospital, for all samples and medical data used in this study have been irreversibly anonymized.

### Patient information

All tumor samples used in the study were collected from the First Affiliated Hospital of Dalian Medical University, China. A total of 93 FFPE tumor samples from colorectal cancer patients were analyzed. Patients ranged from 29-86 years of age with a median age of 63 years (Table 1). The tumor samples were characterized by tissue origin (colon or rectum) and pathologic diagnosis (apophysis or ulcerative) (Table 2). Additional differentiation and TNM staging information is provided in Tables 3-5.

| Characteristic                  | All patients (n=93) |
|---------------------------------|---------------------|
| Age                             |                     |
| Median (years)                  | 62                  |
| Range (years)                   | 29-86               |
| < 60 years: n (%)               | 36 (38.7%)          |
| ≥ 60 years: n (%)               | 57 (61.3%)          |
| Gender                          |                     |
| Male: n (%)                     | 43 (46.2%)          |
| Female: n (%)                   | 50 (53.8%)          |
| Pathologic Type                 |                     |
| Apophysis: n (%)                | 18 (19.4%)          |
| Ulcerative: n (%)               | 68 (73.1%)          |
| Apophysis and Ulcerative: n (%) | 1 (1.1%)            |
| Unknown: n (%)                  | 6 (6.5%)            |
| Pathologic diagnosis location   |                     |
| Rectum: n (%)                   | 81 (87.1%)          |
| Colon: n (%)                    | 11 (11.8%)          |
| Rectum and Colon: n (%)         | 1 (1.1%)            |

**Table 1:** Patients' characteristics for 93 human rectum and colon cancer samples.

| Gene   | Total number of samples with mutation in 93 samples (%) | Sex  |  | Location                                      |  |  | Type  |  |  |  |
|--------|---|--|--|---|--|--|---|--|--|--|
|        |   | Male samples with mutation in 43 samples (%) | Female samples with mutation in 50 samples (%) | Colon samples with mutation in 11 samples (%) | Rectum samples with mutation in 81 samples (%) | Colon/Rectum samples with mutation in 1 sample (%) | Apophysis samples with mutation (% in 18 samples) | Ulcerative samples with mutation (% in 68 samples) | Apophysis/Ulcerative samples with mutation (% in 1 sample) | Unknown samples with mutation (% in 6 samples) |
| APC    | 16 (17.2%)  | 7 (16.3%)                                    | 9 (18.0%)                                      | 1 (9.1%)                                      | 15 (18.5%)                                     | 0 (0.0%)   | 2 (11.1%)   | 14 (20.6%)   | 0 (0.0%)   | 0 (0.0%)                                       |
| BRAF   | 1 (1.1%)  | 0 (0.0%)                                     | 1 (2.0%)                                       | 0 (0.0%)                                      | 1 (1.2%)                                       | 0 (0.0%)   | 0 (0.0%)  | 1 (1.5%)   | 0 (0.0%)   | 0 (0.0%)                                       |
| CTNNB1 | 1 (1.1%)  | 0 (0.0%)                                     | 1 (2.0%)                                       | 0 (0.0%)                                      | 1 (1.2%)                                       | 0 (0.0%)   | 0 (0.0%)  | 1 (1.5%)   | 0 (0.0%)   | 0 (0.0%)                                       |
| FBXW7  | 10 (10.8%)  | 7 (16.3%)                                    | 3 (6.0%)                                       | 2 (18.2%)                                     | 8 (9.9%)                                       | 0 (0.0%)   | 3 (16.7%)   | 7 (10.3%)  | 0 (0.0%)   | 0 (0.0%)                                       |
| KRAS   | 47 (50.5%)  | 22 (51.2%)                                   | 25 (50.0%)                                     | 6 (54.5%)                                     | 40 (49.4%)                                     | 1 (100.0%)   | 9 (50.0%)   | 33 (48.5%)   | 1 (100.0%)   | 4 (66.7%)                                      |
| NRAS   | 3 (3.2%)  | 1 (2.3%)                                     | 2 (4.0%)                                       | 0 (0.0%)                                      | 3 (3.7%)                                       | 0 (0.0%)   | 1 (5.6%)  | 2 (2.9%)   | 0 (0.0%)   | 0 (0.0%)                                       |
| PIK3CA | 10 (10.8%)  | 7 (16.3%)                                    | 3 (6.0%)                                       | 1 (9.1%)                                      | 8 (9.9%)                                       | 1 (100.0%)   | 3 (16.7%)   | 6 (8.8%)   | 1 (100.0%)   | 0 (0.0%)                                       |
| SMAD4  | 2 (2.2%)  | 1 (2.3%)                                     | 1 (2.0%)                                       | 0 (0.0%)                                      | 2 (2.5%)                                       | 0 (0.0%)   | 1 (5.6%)  | 1 (1.5%)   | 0 (0.0%)   | 0 (0.0%)                                       |
| TP53   | 22 (23.7%)  | 8 (18.6%)                                    | 14 (28.0%)                                     | 3 (27.3%)                                     | 18 (22.2%)                                     | 1 (100.0%)   | 7 (38.9%)   | 13 (19.1%)   | 1 (100.0%)   | 1 (16.7%)                                      |

**Table 2:** Detected mutations frequencies of 93 human rectum and colon cancers based on sex, diagnostic location (colon vs. rectum), and type (apophysis vs. ulcerative).

### DNA preparation

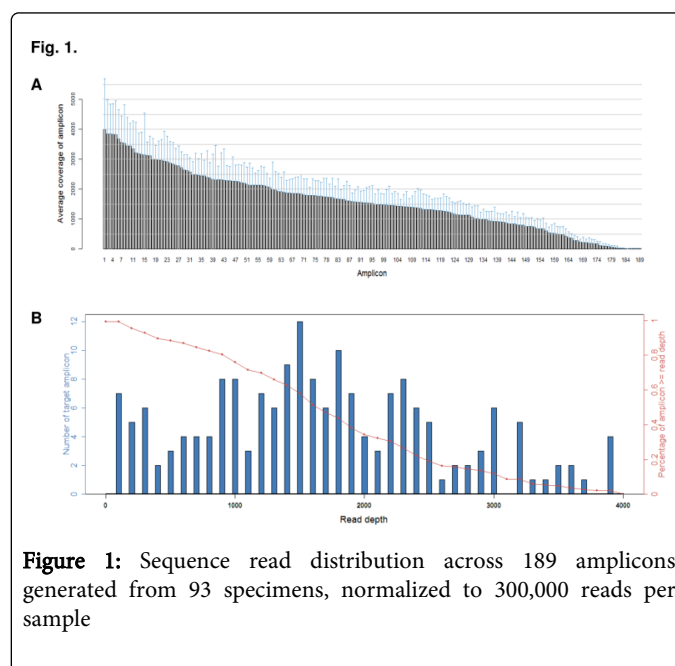
Formalin-fixed, paraffin embedded (FFPE) tissue samples were deparaffinized in xylene prior to extraction of 3 – 5 μm thick sections. The QIAamp DNA Mini Kit (Qiagen) was then used to isolate DNA as per the manufacturer’s instructions.

### Ion torrent PGM library preparation and sequencing

An Ion Torrent adapter-ligated library was constructed with the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Part #4475345 Rev. A) following the manufacturer’s protocol, and as in our previous publications [16,17]. The Personalized Cancer Mutation Panel used for this study targets 737 loci to detect mutations in the following 45 cancer-related genes: ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNAS, HNF1A, HRAS, IDH1, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, and VHL.

### Sequence coverage

For the 93 CRC samples analyzed, the mean read length was 76 bp and the average sequence per sample was approximate 18 Mb. With normalization to 300,000 reads per specimen, there was an average of 1,639 reads per amplicon (range: 15 to 3,979) (Figure 1A); 176/189 (93.1%) amplicons averaged at least 100 reads, and 165/189 (87.3%) amplicons averaged at least 300 reads (Figure 1B).



**Figure 1:** Sequence read distribution across 189 amplicons generated from 93 specimens, normalized to 300,000 reads per sample. Average number of reads observed for each amplicon. Number of targets with a given read depth, sorted in bins of 100 reads. (blue bar means Number of targets within read depth, red line means % of targets ≥ read depth)

### Variant calling

The Ion Torrent platform-specific pipeline software Torrent Suite initially processed data from the PGM runs to generate sequence

reads, trim adapter sequences, filter, and remove any poor signal-profile reads. Initial variant calling from the Ion AmpliSeq sequencing data was performed with Torrent Suite Software v3.0 with a plug-in “variant caller” program. With the aim of eliminating erroneous base calling, several filtering steps were utilized to produce final variant calling, including defining variant frequency and coverage depth, eliminating DNA strand-specific errors, defining variants within 727 hotspots, and removing variants in amplicon AMPL339432 (PIK3CA, exon13, chr3:178938822-178938906), and as further detailed in our previous publications [16,17]. Additionally, as the JAK2 gene locus generated false deletion data, sequencing data from this locus was excluded from further analysis. Furthermore, Sanger’s sequencing was frequently used to confirm the detected missense mutations.

### Somatic mutations

Detected mutations were compared to variants in the 1000 Genomes Project [18] and 6,500 exomes of the National Heart, Lung, and Blood Institute Exome Sequencing Project [19] to distinguish somatic mutations and germline mutations.

### Bioinformatical and experimental validation

We used the COSMIC (version 64) [20], My Cancer Genome database (<http://www.mycancergenome.org/>), and additional publications to evaluate reappearing mutations in CRCs (Supplemental Table 1). Additionally, some of the detected mutations were confirmed by Sanger’s sequencing (Supplemental Table 2).

## Results and Discussion

### Mutation analysis of human colorectal cancers with ion ampliseq cancer panel

A total of 93 human colon and rectal cancer samples were analyzed using Ion Torrent Ampliseq Cancer Panel to identify mutations in 737

loci from 45 oncogenes and tumor suppressor genes. The sequenced data were processed and we found frequent mutations in the following genes: APC (17.2%), FBXW7 (11.8%), KRAS (51.6%), PIK3CA (10.8%), and TP53 (23.7%). Mutations were also identified in the following genes at lesser frequencies: BRAF (1.1%), CTNNB1 (1.1%), NRAS (3.2%), and SMAD4 (2.2%). When sufficient DNA was available, Sanger’s sequencing was used to confirm these mutations, and for those samples, Sanger’s sequencing confirmed 100% of these mutations (40/40) (Supplemental Table 2) (Supplementary Figure 1-40).

Overall, mutations were found in 70 of the 93 samples (75.3%). Specifically, 38 (40.9%) samples contained one mutation (Table 3), 20 (21.5%) had two mutations (Table 4), and 12 (12.9%) had three mutations (Table 5) among the 45 genes screened. The number of mutations found per sample tends to correlate with staging of the cancer: for samples with one mutation and reported staging, 30% were stage 3; for samples with two mutations and reported staging, 66.7% were stage 3 or higher; and for samples with three mutations and reported staging, 77.8% were stage 3 or higher. 81.8% of colon samples had at least one mutation, whereas 74.1% of rectal samples had at least one mutation. Proportionally, the APC mutation rate was twice as high in rectal samples vs. those from the colon, which is consistent with CRC COSMIC data [21], whereas the opposite was true for FBXW7 mutations. The other mutations were found at roughly equal proportions between pathological locations. Mutations were also found to be proportionally similar between males and females, with the exception of FBXW7 (18.6% vs. 6.0%, respectively), PIK3CA (16.3% vs. 6.0%, respectively), and TP53 (18.6% vs. 28.0%, respectively) (Table 2).

| Gene   | Mutation | Sex | Age | Location | Type       | Differentiation | TNM Stage |
|--------|----------|-----|-----|----------|------------|-----------------|-----------|
| APC    | Q1303*   | F   | 57  | Rectum   | Ulcerative | Middle          | ND        |
| APC    | E1306*   | F   | 64  | Rectum   | Ulcerative | Middle-Low      | ND        |
| APC    | Q1367*   | M   | 58  | Rectum   | Ulcerative | Middle-High     | ND        |
| APC    | R1450*   | M   | 64  | Colon    | Ulcerative | Middle-Low      | ND        |
| BRAF   | V600E    | F   | 79  | Rectum   | Ulcerative | Middle          | ND        |
| CTNNB1 | S45F     | F   | 56  | Rectum   | Ulcerative | Middle          | I         |
| FBXW7  | R505C    | F   | 57  | Rectum   | Ulcerative | Middle          | ND        |
| KRAS   | G12C     | M   | 59  | Rectum   | Unknown    | Middle-High     | II        |
| KRAS   | G12D     | M   | 54  | Colon    | Unknown    | Middle          | ND        |
| KRAS   | G12D     | F   | 68  | Rectum   | Ulcerative | Middle          | ND        |
| KRAS   | G12D     | F   | 42  | Rectum   | Unknown    | Middle          | I         |
| KRAS   | G12D     | M   | 68  | Rectum   | Ulcerative | Middle          | III       |
| KRAS   | G12D     | F   | 60  | Rectum   | Apophysis  | Middle          | ND        |

|        |       |   |    |        |            |             |     |
|--------|-------|---|----|--------|------------|-------------|-----|
| KRAS   | G12D  | M | 82 | Rectum | Ulcerative | Middle      | I   |
| KRAS   | G12D  | F | 78 | Rectum | Ulcerative | Middle      | ND  |
| KRAS   | G12D  | M | 58 | Rectum | Ulcerative | Middle-High | III |
| KRAS   | G12D  | F | 63 | Rectum | Ulcerative | High        | II  |
| KRAS   | G12S  | F | 72 | Colon  | Ulcerative | Middle      | III |
| KRAS   | G12V  | M | 49 | Rectum | Ulcerative | Middle-High | II  |
| KRAS   | G12V  | F | 67 | Rectum | Ulcerative | Unknown     | ND  |
| KRAS   | G13D  | F | 75 | Rectum | Ulcerative | Low         | II  |
| KRAS   | G13D  | F | 58 | Rectum | Ulcerative | Middle      | ND  |
| KRAS   | G13D  | F | 70 | Rectum | Ulcerative | Middle      | II  |
| KRAS   | G13D  | M | 62 | Rectum | Apophysis  | Middle      | II  |
| KRAS   | G13D  | F | 53 | Rectum | Ulcerative | Middle-High | III |
| KRAS   | G13D  | M | 86 | Colon  | Ulcerative | Middle-High | II  |
| KRAS   | G13D  | F | 71 | Rectum | Unknown    | High        | III |
| KRAS   | G13D  | F | 49 | Rectum | Ulcerative | Unknown     | ND  |
| KRAS   | A146T | M | 60 | Rectum | Ulcerative | Middle      | ND  |
| NRAS   | Q61K  | F | 67 | Rectum | Apophysis  | Middle      | I   |
| NRAS   | Q61R  | M | 49 | Rectum | Ulcerative | Middle      | III |
| PIK3CA | E545K | M | 50 | Colon  | Ulcerative | Middle-High | ND  |
| TP53   | Y163C | F | 59 | Rectum | Apophysis  | Middle      | ND  |
| TP53   | R175H | F | 67 | Colon  | Apophysis  | Middle-High | II  |
| TP53   | R175H | M | 62 | Rectum | Ulcerative | Middle-High | ND  |
| TP53   | C176F | F | 72 | Rectum | Unknown    | Middle      | ND  |
| TP53   | Y220C | F | 60 | Rectum | Ulcerative | Middle      | II  |
| TP53   | C275Y | M | 64 | Rectum | Apophysis  | Middle      | I   |

**Table 3:** Single point mutations detected among 93 colorectal cancer samples.

\*Nonsense mutations resulting in STOP codon; ND: Not determined

| Gene 1 | Mutation 1 | Gene 2 | Mutation 2 | Sex | Age | Location | Type       | Differentiation | TNM Stage |
|--------|------------|--------|------------|-----|-----|----------|------------|-----------------|-----------|
| KRAS   | G12D       | APC    | R876*      | M   | 79  | Rectum   | Ulcerative | Middle          | ND        |
| KRAS   | G12D       | APC    | R876*      | F   | 79  | Rectum   | Ulcerative | Middle          | II        |
| KRAS   | G12D       | APC    | E1309fs*4  | F   | UNK | Rectum   | Ulcerative | High            | II        |
| KRAS   | G13D       | APC    | Q1378*     | M   | 70  | Rectum   | Ulcerative | Middle-High     | III       |
| KRAS   | G13D       | APC    | R1450*     | F   | 76  | Rectum   | Apophysis  | Middle          | ND        |
| KRAS   | G12D       | TP53   | R158H      | F   | 81  | Rectum   | Ulcerative | Middle          | III       |

|       |       |        |        |   |    |        |            |            |     |
|-------|-------|--------|--------|---|----|--------|------------|------------|-----|
| KRAS  | G12D  | TP53   | Y220C  | F | 45 | Rectum | Ulcerative | Middle     | IV  |
| KRAS  | G12V  | TP53   | F270S  | M | 66 | Colon  | Apophysis  | Middle-Low | II  |
| KRAS  | G13D  | TP53   | V173L  | F | 58 | Rectum | Ulcerative | Middle-Low | III |
| KRAS  | G13D  | TP53   | R248W  | F | 49 | Rectum | Ulcerative | Middle     | ND  |
| KRAS  | G12D  | PIK3CA | E545K  | F | 57 | Rectum | Apophysis  | Middle-Low | III |
| KRAS  | G12V  | PIK3CA | E545K  | M | 69 | Rectum | Ulcerative | Middle     | ND  |
| KRAS  | G12V  | PIK3CA | E545K  | M | 61 | Rectum | Ulcerative | Low        | ND  |
| KRAS  | G12C  | FBXW7  | R465C  | M | 63 | Colon  | Ulcerative | Middle     | ND  |
| TP53  | R273C | APC    | Q1367* | M | 63 | Rectum | Ulcerative | Middle     | III |
| TP53  | E285K | APC    | Q1367* | F | 53 | Rectum | Apophysis  | Middle     | III |
| TP53  | A159V | NRAS   | G13R   | F | 58 | Rectum | Ulcerative | Middle     | III |
| TP53  | G245V | SMAD4  | Q245*  | F | 65 | Rectum | Ulcerative | Middle     | ND  |
| FBXW7 | R465H | APC    | K1370* | F | 51 | Rectum | Ulcerative | Middle-Low | II  |
| FBXW7 | R465C | PIK3CA | H1047R | M | 79 | Rectum | Ulcerative | Middle     | III |

**Table 4:** Combination point mutations in two genes detected among 93 colorectal cancer samples.

\*Nonsense mutations resulting in STOP codon; fs: Frameshift mutation; ND: Not determined

| Gene 1 | Mutation 1 | Gene 2 | Mutation 2 | Gene 3 | Mutation 3 | Sex | Age | Location | Type       | Differentiation | TNM Stage |
|--------|------------|--------|------------|--------|------------|-----|-----|----------|------------|-----------------|-----------|
| KRAS   | G12D       | TP53   | R175H      | APC    | Q1303*     | F   | 76  | Rectum   | Ulcerative | Middle          | III       |
| KRAS   | G12D       | TP53   | R273C      | APC    | E1309fs*4  | F   | 75  | Rectum   | Ulcerative | Middle          | II        |
| KRAS   | G12V       | TP53   | R248W      | FBXW7  | R465C      | M   | 59  | Rectum   | Apophysis  | Middle          | II        |
| KRAS   | A146T      | TP53   | P152L      | FBXW7  | R505L      | M   | 80  | Colon    | Ulcerative | Middle          | III       |
| KRAS   | G12D       | TP53   | R213*      | PIK3CA | E545K      | M   | 56  | Rectum   | Apophysis  | Middle          | ND        |
| KRAS   | G12D       | TP53   | E298*      | PIK3CA | E542K      | M   | 67  | Both     | Both       | Unknown         | III       |
| KRAS   | G13D       | TP53   | R175H      | PIK3CA | M1043V     | F   | 29  | Rectum   | Ulcerative | Mid-High        | III       |
| KRAS   | G12D       | PIK3CA | E545K      | FBXW7  | R278*      | F   | 67  | Rectum   | Apophysis  | Middle          | III       |
| KRAS   | G12S       | PIK3CA | E545K      | FBXW7  | R479Q      | M   | 73  | Rectum   | Ulcerative | Middle          | III       |
| KRAS   | G13A       | KRAS   | G13R       | APC    | R1114*     | M   | 77  | Rectum   | Ulcerative | Middle          | ND        |
| KRAS   | G12D       | SMAD4  | R361H      | FBXW7  | R465H      | M   | 57  | Rectum   | Apophysis  | Middle          | ND        |
| FBXW7  | R278*      | FBXW7  | R465H      | APC    | K1370*     | M   | 67  | Rectum   | Ulcerative | Middle          | IV        |

**Table 5:** Combination point mutations in three genes detected among 93 colorectal cancer samples.

\*Nonsense mutations resulting in STOP codon; fs: Frameshift mutation; ND: Not determined

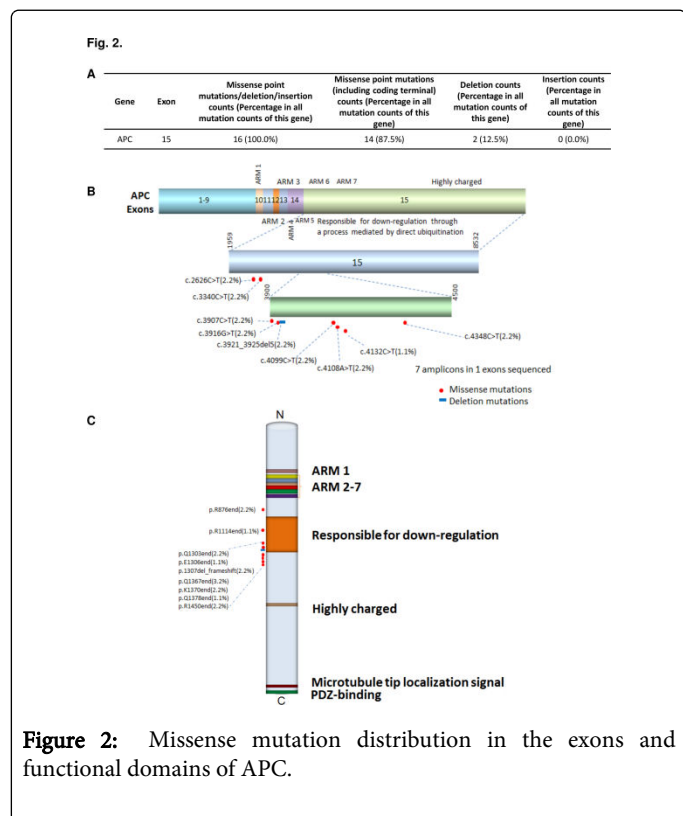
#### APC mutations

Of the 93 sequenced colorectal cancer samples, 16 APC mutations were found, all of which were located in exon 15 (Figure 2A). Fourteen

of the 16 mutations detected resulted in a stop codon (R876\*, R1114\*, Q1303\*, E1306\*, Q1367\*, Q1378\*, R1450\*) and truncated protein product, and the other two mutations were deletions resulting in a frameshift (E1309fs\*4) (Figure 2B and 2C). The APC gene consists of 21 exons and encodes a 2,843 amino acid protein [22,23]. Exon 15 comprises 77% of the coding sequence of APC and is the most



common target for both germline and somatic mutations [24]. Specifically, a mutation cluster region (MCR) in exon 15 between codons 1,286 and 1,513, which comprises only 10% of the total APC coding sequence, accounts for greater than 90% of APC mutations in all CRCs [25].



**Figure 2:** Missense mutation distribution in the exons and functional domains of APC.

Frequencies of detected mutations in different exons.

Mutation distribution in exons.

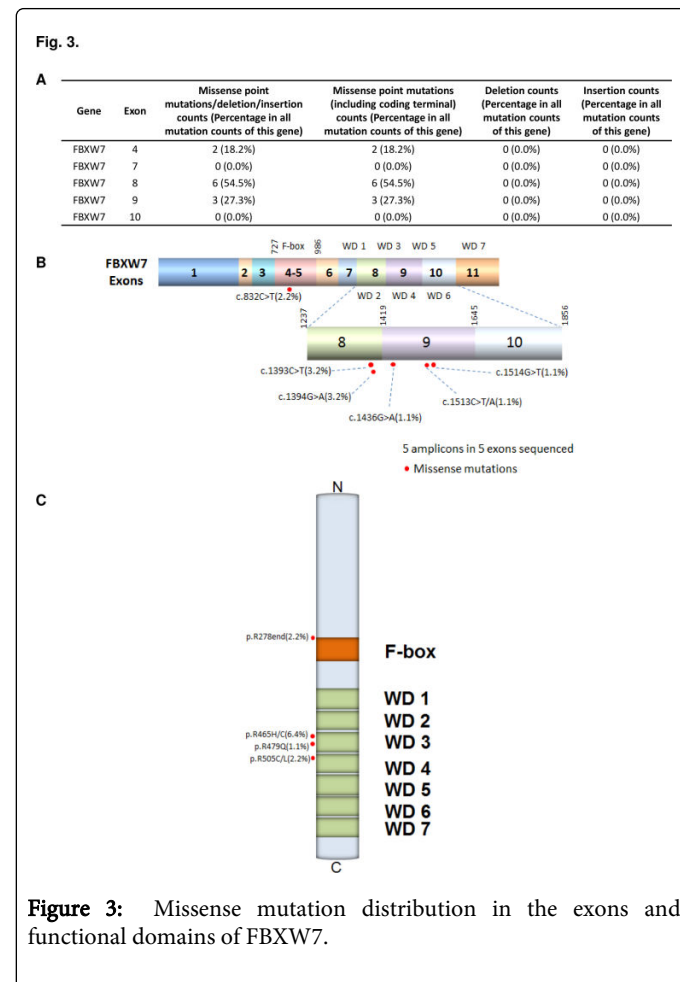
Mutation distribution in functional domains.

APC is an important tumor suppressor known to play a role in colorectal cancer. Mutations in the APC gene leads to a condition known as familial adenomatous polyposis (FAP), in which the intestinal epithelium is studded with hundreds to thousands of benign polyps, some of which may progress to invasive cancers if left untreated [26]. The vast majority of FAP-associated APC mutations lead to a truncated protein. The protein translated from the APC gene product interacts with cytoplasmic beta-catenin, mediates its degradation through ubiquitination, and thereby downregulates transcription exerted by the  $\beta$ -catenin-Tcf complex [24]. However, in the absence of a functional APC protein,  $\beta$ -catenin is stabilized and accumulates in the cytoplasm [24]. This results in constitutive transcriptional activation of Tcf-responsive genes, which may contribute to cancer progression [24].

### FBXW7 mutations

Eleven FBXW7 mutations were identified in ten samples (10.8%), which is consistent with previous findings that roughly 10% of CRCs have FBXW7 mutations [27].

These mutations were distributed along the following exons: R278<sup>\*</sup> in exon 2 (18.2%), R465C/H in exon 6 (54.5%), and R479Q and R505C/L in exon 9 (27.3%) (Figure 3).



**Figure 3:** Missense mutation distribution in the exons and functional domains of FBXW7.

Frequencies of detected mutations in different exons.

Mutation distribution in exons.

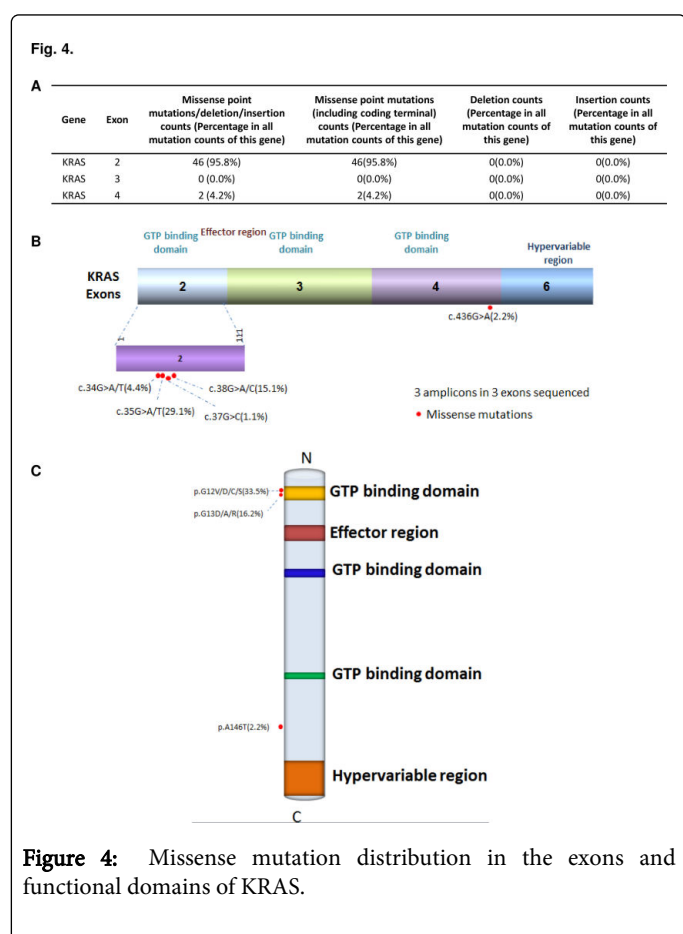
Mutation distribution in functional domains.

FBXW7 (Cdc4) is a potential tumor suppressor that regulates ubiquitination and proteolysis of multiple targets. It negatively regulates multiple proteins with established roles in the control of cell division and growth, including cyclin E, c-Jun, c-Myc, mTOR, and Notch. FBXW7 proteins contain domains called WD4 repeats, which can form eight-bladed, barrel-shaped  $\beta$ -propeller-like binding pockets for substrates [28,29]. Arginine residues at the top of these propeller-like pockets directly interact with the recognition sequences in substrate [28,29], and mutations at codons 465, 479, and 505 lie at the  $\beta$ -propeller tips which interact with FBXW7 substrates [30]. Mutations in FBXW7 impair Cyclin E degradation and are associated with decreased genetic stability and impaired growth regulation, contributing to the progression of CRC [31,32]32.

### KRAS mutations

KRAS mutations have been reported in up to 50% of all human CRCs [33]. In accordance, 47 of the samples in our study (50.5%)

contained at least one KRAS mutation affecting the GTP binding domains, where 90.2% of these mutations were missense in exon 2 (G12C/D/S/V and G13A/D/R), and 4.2% were found in exon 4 (A146T) (Figure 4). Consistent with our results, roughly 90% of all KRAS mutations in human cancers occur in codon 12 and 13, whereas the A146 mutation occurs in approximately 4% of CRCs [34]. These mutations are thought to occur early in carcinogenesis between the transitions from benign to late stage adenomas [34]. Various CRC studies suggest that KRAS mutations in codons 12 and 13 are generally predictive of a poorer prognosis [35], whereas mutations in exon 4 are associated with a more favorable clinical outcome in CRC patients [36].



Frequencies of detected mutations in different exons.

Mutation distribution in exons.

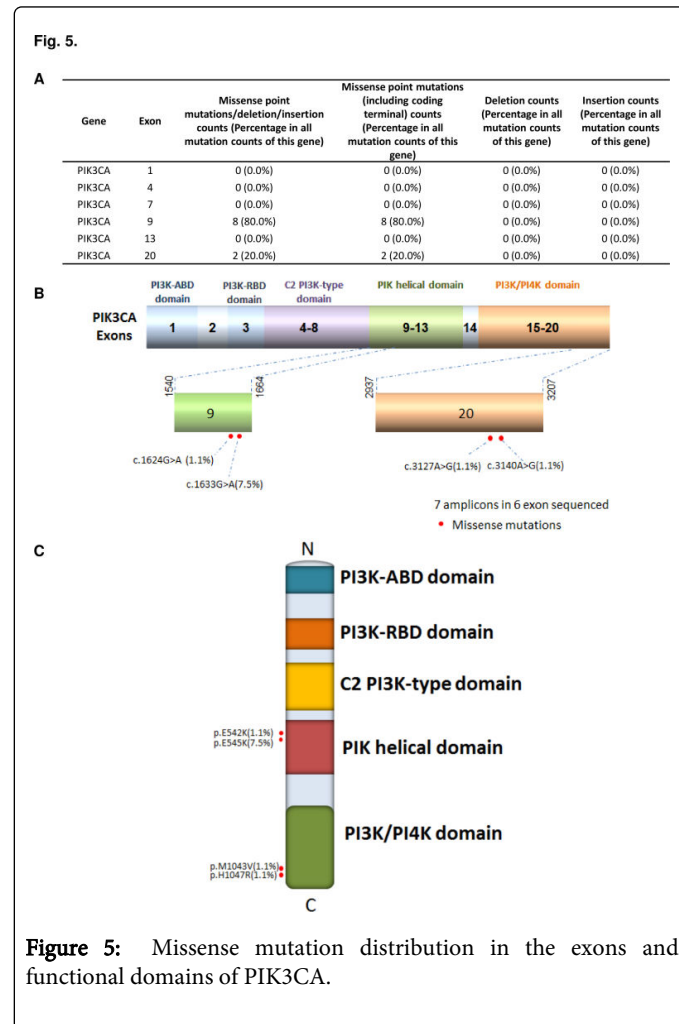
Mutation distribution in functional domains.

KRAS is an oncogene involved in the MAPK signaling cascade, and KRAS mutations have been shown to promote adenoma growth in the progression of CRC development. KRAS binds to GTP in the active state and possesses an intrinsic enzymatic activity which cleaves the terminal phosphate of the nucleotide and converts it to GDP. Upon conversion of GTP to GDP, KRAS is turned off [37], and the result of KRAS mutations is constitutive activation of KRAS signaling pathways. Once turned on, KRAS recruits and activates proteins necessary for the propagation of growth factor and other receptors' signal, such as c-Raf and PI3-kinase [37]. Thus early KRAS mutations

influence the signal transduction process which promotes intestinal adenomatous growth [33].

### PIK3CA mutations

An estimated 10-30% of all CRCs have PIK3CA mutations [38]. Accordingly, ten of our 93 CRC samples (10.8%) harbored missense mutations in the PIK3CA gene (Figure 5A), where 80.0% were found in exon 9 (E542K and E545K) and 20.0% in exon 20 (M1043V and H1047R). These exons encode for the helical and kinase domain of PI3K, respectively (Figure 5B and 5C), and roughly 80% of oncogenic PIK3CA mutations are located in these mutation hotspots [39].



Frequencies of detected mutations in different exons.

Mutation distribution in exons.

Mutation distribution in functional domains.

PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) is a lipid kinase capable of phosphorylating the 3'OH of the inositol ring of phosphoinositides. PI3K is responsible for coordinating a diverse range of cellular functions including proliferation, migration, and cell survival, as well as oncogenic transformations. Mutations in the kinase and helical domains of PI3K is associated with increased lipid kinase activity, and activation of



downstream Akt signaling caused by these mutations interferes with other signaling pathways that contribute to oncogenicity [40-42].

### TP53 mutations

TP53 incurred several mutations across multiple exons in our sample set: 45.5% in exon 5 (P152L, R158H, A159V, Y163C, V173L/G, R175H, and C176F), 13.6% in exon 6 (R213\* and Y220C), 13.6% in exon 7 (G245V and R248W), and 27.3% in exon 8 (F270S, R273C, C275Y, C277F, E285K, and E298\*) (Figure 6). Most of these mutations are at known hotspot mutation sites, with the exception of A159V, F270S, C277F, E285K, and E298\*, which are common mutations, and V173G, which is not common. All of these mutations occurred in the DNA binding region, and some of these occurred in the L2-L3 region (V173L/G, R175H, C176F, G245V, and R248W). Consistent with our findings, most TP53 mutations cluster in the TP53 DNA-binding domain, which encompasses exons 5 through 8 and spans approximately 180 codons [43]. Our sample set had a lower TP53 mutation frequency than expected (23.7%), as previous studies have found TP53 mutations in 50-70% of colorectal cancers [44], which may reflect differences in populations or our limited sample size.

Abnormality of the TP53 gene is one of the most common events in CRCs and plays an important role in the tumorigenesis of colorectal epithelial cells. TP53 mutations are thought to be relatively late events in CRC development, with a loss of TP53-mediated apoptotic pathways as an important factor in the progression from an adenoma to a malignant tumor [25]. Most TP53 missense mutations lead to the synthesis of a stable protein, but this lacks specific DNA-binding and transactivation functions and accumulates in the nucleus of cells. Such mutant proteins become inactive and lose the ability to transactivate downstream target genes that regulate cell cycle and apoptosis [45]. Apart from these mutations affecting the role of TP53 as a tumor-suppressor protein, TP53 mutations also endow the mutant protein with 'gain-of-function' (GOF) activities, which can contribute actively to various stages of tumor progression, including distant metastases and an increased resistance to anticancer treatments [46,47].

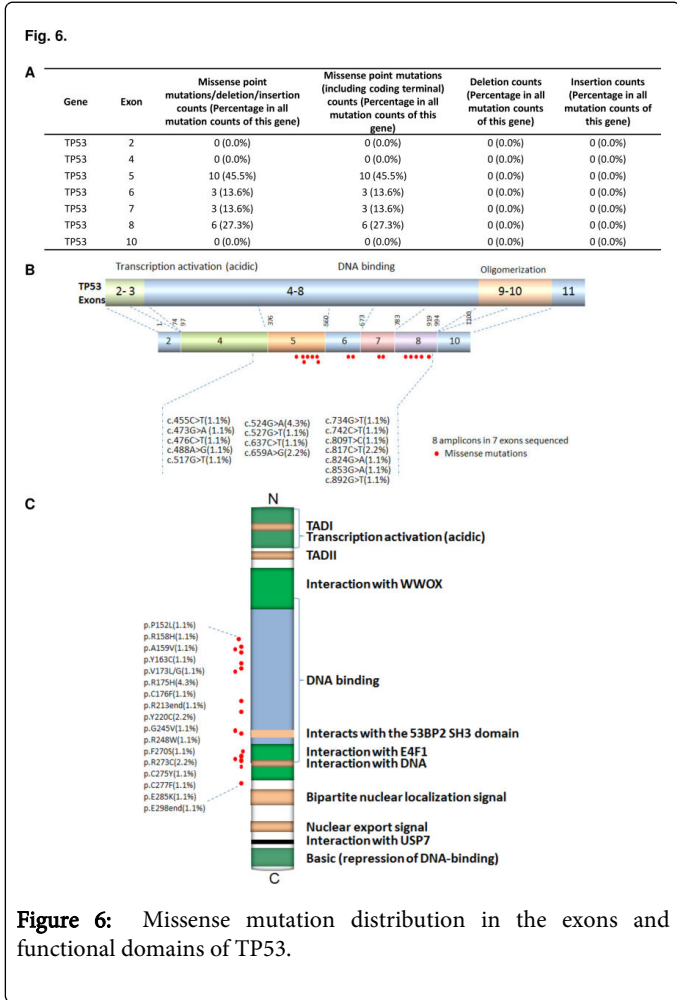
### Combination mutations

Roughly one third of the CRC samples (34.4%) contained more than one mutation, where 21.5% harbored two mutations (Table 4) and 12.9% harbored three mutations (Table 5). Of the 70 samples with mutations, 63 (90.0%) had a mutation in APC, KRAS, and/or TP53; 17.1% had KRAS and TP53 mutations, 11.4% had a combination of KRAS and PIK3CA or KRAS and APC, 8.6% with KRAS and FBXW7, and 5.7% with APC and TP53. Not only were KRAS and TP53 mutations found at the highest frequency in our study, these mutations were also found most commonly in combination with mutations in other genes: 14/20 (70.0%) of samples with two mutations had a KRAS mutation, and 9/20 (45.0%) harbored a TP53 mutation. Of the double mutants, 25% were a combination of KRAS and TP53, and 25% were KRAS and APC. Interestingly, all but one sample (91.7%) with three mutations had at least one KRAS mutation, and 50% of these samples contained at least one FBXW7 mutation. Three samples (25.0%) contained a combination of KRAS, TP53, and PIK3CA.

A previous study on gene mutations in European CRC patients found the most common combination mutations to be TP53 and APC, whereas mutations in TP53 co-occurring with KRAS were rare [25]; conversely, we found TP53 and APC combination mutations in only 4.3% of our samples and TP53 plus KRAS combination mutations in 12.9% of CRCs. Another study with Taiwanese CRC samples found the most common combination mutations to occur between APC and KRAS [48], whereas we identified that combination in only 8.6% of our samples. Taken together, these results indicate the wide variability in genetic mutations found in CRCs from different populations and underscore the need to further evaluate CRCs for common mutation patterns.

### Conclusion and future directions

In the present study, we used Ion Torrent Ampliseq Cancer Panel to sequence 737 loci from 45 cancer-related genes, mainly oncogenes and tumor suppressor genes, in 93 human CRC samples. We identified frequent mutations in APC, FBXW7, KRAS, PIK3CA, and TP53 (Table 2), and the mutation profile was consistent with prior knowledge of genetic alterations in colorectal cancer [49]. In comparison with traditional Sanger sequencing and other Next Generation Sequencing (NGS) methods which utilize a 4-color optical detection system, the Ion Torrent sequencing platform has a major advantage in its Post-Light sequencing technology, which reduces both assay cost and complexity. This technology allows Ion Torrent



**Figure 6:** Missense mutation distribution in the exons and functional domains of TP53.

Frequencies of detected mutations in different exons.

Mutation distribution in exons.

Mutation distribution in functional domains.

sequencing to produce reliable results at a relatively faster rate and reduced sequencing costs per base [50].

In developed countries, colorectal cancer is the third most common malignancy and the second most frequent cause of cancer-related death. Additionally, prognosis is poor for patients with this disease and the overall 5-year survival rate is only 6.6%–11.9% [51], indicating that the traditional treatments of surgery, radiotherapy, and generalized chemotherapy are less than optimal. Hence, treatment of CRC has been quite a challenge for clinicians, and targeting specific molecular pathways and gene mutations has become a critical step in improving treatment options and increasing patient survival. Therefore, identifying and understanding the mutation profile of individual cancer genomes may provide valuable information on targeted treatments and potential routes for treatment.

A number of targeted agents have been developed, including EGFR, VEGF, and multi-kinase inhibitors, which in combination with chemotherapy have demonstrated improved outcome in metastatic colorectal cancer patients [52,53]. In addition to developing targeted agents to specific mutations, it is also important to understand their molecular action thoroughly in order to avoid any side- and counter-effects. For example, patients harboring KRAS mutations are resistant to treatment with anti-EGFR monoclonal antibodies. Consistently, recent clinical evidences from phase II and phase III trials of Panitumumab and Cetuximab showed that patients with KRAS mutations in their tumors did not respond appropriately to the targeted drugs [54]. Currently KRAS mutation testing is routinely performed to prevent patients' exposure to unnecessary drug toxicities in situations where anti-EGFR monoclonal antibodies would not be beneficial. However, there are currently no specific treatment options for patients harboring KRAS mutations, and as KRAS is the most frequently mutated gene in CRCs, these patients are in critical need for alternative target therapies.

Accumulating evidence indicates that effective treatment for the majority of most malignancies requires more than the administration of a single agent but a combination of multiple therapies [55]. By identifying mutation combinations in individual cancers, such as in our study, directed treatments with a combination of targeted agents against the detected mutations may prove to have greater benefits for cancer patients. Ion Torrent sequencing may help provide clinicians a rapid, affordable, and feasible way to reliably identify mutations in CRCs to further improve patient treatments and outcomes in the near future.

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