Assessing the Mutational Landscape of BRAF- and KRAS-Wild Type Colorectal Cancer

Assessing the Mutational Landscape of *BRAF*- and *KRAS*-wild type Colorectal Cancer

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Abstract

Colorectal cancer is one of the most deadly cancers and is the second leading cause of death in the United States. As incidence of colorectal cancer is higher among developed countries, research continues in hopes to make marked advancements in the way cancer is treated. A number of targeted therapies have been developed and are in clinical use; increasingly these require genomic analyses as a companion diagnostic or a predictive biomarker. This is the concept of precision cancer therapy: genomic sequencing of patients' tumors to assess for known mutations in a certain cancer type, which allows doctors to prescribe more effective therapeutics based on the drivers of that patient's individual cancer.

Data from genomic studies has shown that over half of colorectal adenocarcinomas are driven by activating mutations in *KRAS* or *BRAF* oncogenes. However, the other 50% of colorectal cancer cases are wild type for both genes; thus identification and characterization of the landscape of mutations in non-*BRAF* and *KRAS*-mutated colorectal cancer is helpful to propose suitable therapies for these patients. This study assesses the mutational landscape, including potential driver events, in cases of colorectal cancer that are wild type for both *KRAS* and *BRAF* mutations. Tumor genomic data from the Profile research initiative from Dana-Farber Cancer Institute, Brigham and Women's Hospital, and Boston Children's Hospital was used for analysis. We describe the landscape of alterations- including mutations, copy number changes, and structural rearrangements- and compare between our two cohorts of colorectal cancer patients.

NRAS and *TP53* mutations, as well as *ERBB2*, *FGFR1*, and *EGFR* amplifications, occurred more frequently in the wild-type cohort than the mutant cohort.

We also assessed the number of cases in the wild-type cohort that would be eligible for clinical trials at Dana-Farber Cancer Institute. Using a matching algorithm, we found that 71% of cases that were wild-type for *BRAF* and *KRAS* were potential candidates for currently available clinical trials. We show that patients that have *KRAS/BRAF* wild-type colorectal cancer have non-standard therapies that may be relevant for treatment and highlight the utility of genomic screening for driver alterations in colorectal cancer patients beyond *KRAS* and *BRAF*.

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Chapter I

Introduction

Colorectal Cancer Background

Colorectal cancer (CRC) is one of the world's most deadly cancers, ranking fourth in incidence after lung, liver and stomach cancer (http://www.genome.gov, 2016). The prognosis for CRC patients is often poor and results in over 700,000 deaths each year (Brenner & Pox, 2014). Studies have indicated that prevalence of CRC is higher among developed nations, with strong ties to a "Western" diet (high in saturated fats and carbohydrates) and lifestyle as major risk factors. In addition, the prevalence of CRC is slightly higher in men than women (4% and 3% respectively) (Brenner & Pox, 2014). Due to its high incidence and poor prognosis, there is an ongoing need to develop better and more effective therapeutic and preventative techniques for this type of cancer.

There are a number of risk factors that are known to be associated with increased risk of developing colon cancer. Such risk factors include age over 50, family history of polyps, family history of colon cancer, genetic alterations, hereditary nonpolyposis colon cancer (HNPCC), familial adenomatous polyposis (FAP), ulcerative colitis or Crohn's disease, personal history of cancer, diet, lifestyle, and smoking (Colon Cancer Alliance, 2017). CRC usually begins as a benign polyp in the colon. From there, it can expand and affect the colon, rectum, and other parts of the digestive system. There are four stages of CRC progression (**Figure 1**). First, cancerous cells begin to form on the inner lining of

the large intestine. Next, the cancer cells imbed and grow in the wall of the colon or lymph vessels. From there, the cancer cells can penetrate the blood or the lymph vessels. Finally, cancer cells spread into the nearby lymph nodes and can be transferred to other sites in the body (http://www.genome.gov, 2016).

Colorectal Cancer Treatment Strategies

Advancements in early detection and regular screening make it possible to prevent CRC by removal of polyps. However, once CRC is diagnosed, it is important to know the stage of cancer in order to determine an appropriate treatment plan. Staging is based on the tumor, node, and metastasis system (TNM) for both colon and rectal cancers. Typically, patients diagnosed in Stage 0 and Stage I have surgery as a treatment option. Stages II, III, and IV can be treated with chemotherapy, biologics, and radiation with or without surgery. See **Table 1** for details on the stages of colorectal cancer. In the past, treatment for metastatic colorectal cancer was largely based on fluoropyrimidine-based chemotherapy (Haan *et al.,* 2014). However, this often resulted in unnecessary toxicity without good results. Cancer treatment is now evolving to a more personalized and targeted approach when possible.

Surgery: Laparoscopy, using a thin, lighted tube, can be useful for detecting and removing abnormal growths and tumors from the colon. Open surgery may be performed to remove a tumor or parts of the colon or rectum. Nearby lymph nodes can also be removed as a part of both types of surgery.

Chemotherapy: Treatment using chemotherapy utilizes different chemicals that can kill cells that undergo rapid cell division. Chemotherapy drugs are usually given in cycles for specific periods of time. Approved chemotherapies for CRC include Lonsurf , CapeOx (Capecitabine and oxaliplatin), FOLFIRI (F-FU, leucovorin and oxaliplatin), Eloxatin (Oxaliplatin), Xeloda (Capecitabine), Camptosar (Irinotecan/CPT-11), 5Fluorouracil (5-FU), and FOLFOXIRI (50FU, leucovorin, oxaliplatin, irinotecan) (Colon Cancer Alliance, 2017).

Biological Therapy: Biologic agents are developed from living sources and can be used to prevent, diagnose, and treat disease. Approved biological therapies for CRC include Cyramza (ramucirumab), Avastin (bevacizumab), Stivarga (regorafenib), Erbitux (cetuximab), and Vectibix (panitumumab). In May 2017, the FDA approved pembrolizumab (KEYTRUDA) for adult and pediatric patients with metastatic, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors that have progressed following prior treatment and have no satisfactory alternative. Pembrolizumab is the first tissue/site agonist to be approved for use in CRC (www.fda.gov, 2017). See **Table 2** for available biological therapies.

Radiation Therapy: Radiation therapy uses high-energy rays to kill cancer cells and is effective only in the treated area. Internal, external, intraoperative, and stereotactic body radiation treatment may be used.

Limitations: There are several limitations to the above described approaches for CRC treatment. Often the stage of the CRC can limit the treatment options for the patient. In addition, the risks of substantial side effects need to be weighed when creating a

treatment plan for a patient. Both chemotherapy and radiation therapy involve the risk of damaging normal cells along with tumor cells.

Personalized Cancer Therapy: The goal of personalized medicine is to individualize medical treatment for each patient based on the patient's specific disease. In order to personalize treatment, doctors need to be able to identify the molecular mechanisms, pathways, or genetic alterations, which drive the disease in that patient.

Targeted therapy works by targeting the specific genes, proteins, or tissues that drive cancer. Most of the targeted therapies that are used are biologics and can be combined with other types of therapy as well.

Precision Cancer Therapy

Precision or "personalized" cancer therapy is the concept of providing individualized cancer treatments that are carefully selected as a result of the patient's specific cancer, and applied at the appropriate point in time of a patient's treatment. The notion of precision cancer medicine arose from the idea that cancer is a disease of the genome: that is, cancer is driven by abnormalities in genes or in gene regulation. These abnormalities, or mutations, can be different in each patient, even those with the same type of cancer. Because these mutations are only present in the tumor cells, and not all body cells, DNA samples from the tumor tissue can be used to analyze and identify these mutations. In many cases, cancer cells become dependent on these alterations; and because these genetic "drivers" are only in tumor cells and not normal cells, there is a potentially exquisitely sensitive therapeutic window in which to treat the cancer and not

harm the other host cells. Hallmark examples of this paradigm are the presence of *BCR-ABL* (Philadelphia chromosome) in chronic myelogenous leukemia (CML) or *KIT* mutations in gastrointestinal stromal tumors (GIST), both targeted by treatment with imatinib. The identification of epidermal growth factor receptor (*EFGR*) mutations in NSCLC strengthened this paradigm (Pao *et al.,* 2004; , Paez *et al.,* 2004; Lynch *et al.,* 2004), and the field of cancer research and treatment began to think about treating cancers by targeting their driver mutations, rather than solely treating by histology/site of origin.

In efforts to improve patient outcomes and healthcare costs, the use of predictive biomarkers for drug therapy and precision medicine has become an active area of research. Precision cancer therapy utilizes DNA sequencing (among other technologies) to characterize the tumor genome. Uncovering the patient's specific tumor profile can assist (along with standard histological and pathological tests) in diagnosis of the type of cancer, but can also help prescribe an appropriate targeted therapy. For example, overexpression of human epidermal growth factor receptor 2 protein (*ERBB2*, also known as *HER*-2) in certain breast cancers can be treated with a *HER*-2 targeted therapy, trastuzumab. Similarly, a mutation in the gene *BRAF* (specifically *BRAF* V600E) can be targeted with vemurafenib in certain melanomas. In addition, a tumor profile can also aid in identifying patients that may not respond to certain therapies. An example of this can be seen in colorectal cancer patients that harbor mutations in the *KRAS* gene. These patients will not respond well to anti-EFGR therapy. *BRAF* mutations usually indicate a poor prognosis and the effectiveness of anti-EGFR therapies with *BRAF* mutations

remains unclear and less predictable (Barras, 2015). The overarching principle is treating the right patient with the right drug at the right time.

Colorectal Cancer Genetics

There are several genetic and epigenetic modifications that regulate proliferation, apoptosis, and angiogenesis, and thus, are involved in the pathogenesis of colorectal cancer. *EGFR* signaling cascade activation is one of the most well described pathways leading to tumorigenesis. Mutations in the oncogenes Kristin rat sarcoma 2 viral oncogene (*KRAS*) and v-RAF murine sarcoma viral oncogene homolog B (*BRAF*) which are located downstream of *EGFR*, play a role in the ultimate activation of the mitogen activated protein kinase (MAPK) pathway (**Figure 2**), which then promotes cell growth and survival (Brenner & Pox, 2014). Colorectal cancer has historically been characterized as being driven by *KRAS* or *BRAF* oncogene mutations (Morkel *et al.,* 2015): *KRAS* mutations are found in 35 to 40% of colon cancers (Gonsalves *et al.,* 2014), and mutations in the *BRAF* oncogene, (especially V600E, in which valine is substituted by glutamate at codon 600) account for an additional 10% of all colorectal cancers (Barras, 2015).

Although mutations in *KRAS* and *BRAF* have been characterized in the context of colorectal cancer, people that are wild type for both genes can still develop this cancer. This indicates that there are additional molecular events associated with colon carcinogenesis. Loss of function mutations in the adenomatous polyposis coli (*APC*) gene is among the earliest events in colorectal tumor progression (Gonsalves *et al.,* 2014).

Although the majority of colorectal cancer cases are sporadic, roughly 5% have a hereditary inheritance pattern. There are two types of colon cancers that can be genetically described in this manner. Familial adenomatous polyposis involves loss of function mutations on the *APC* gene and hereditary nonpolyposis colon cancer (Lynch Syndrome) is characterized by alterations in *MSH2*, *MLH1*, *PMS2*, *MSH6*, or *PMS1*. In addition, mutations in genes *FBXW7*, *TP53* or *PI3KCA* pathway genes are also common in sporadic CRCs (http://www.genome.gov, 2016).

Additional, rarer mutational events have also been described in CRC. Among these are driver mutations in *ERBB2, COL12A1, MLL2, FAT4M* and *ARID1A* (Foo *et al.,* 2015). TCGA identified frequent mutations in *TP53, APC, PIK3CA, KRAS, BRAF*, and *SOX9* among others. In addition, certain genes have been described as drug targets and may be found in CRC, such as *FGFR1* overexpression (treatment with pazopanib or regorafenib), *FLT3* amplification (treatment with sorafenib and sunitinib), and *VEGFA* overexpression (treatment with bevacizumab, ranibizumab, aflibercept, and pegaptanib).

NCCN guidelines for the treatment of CRC indicate that all patients with metastatic colorectal cancer should have tissue genotypes for RAS genes, including *KRAS* and *NRAS*, and *BRAF* mutations. Patients with any known *KRAS* mutations (exon 2 or non-exon 2) or *NRAS* mutations should not be treated with cetuximab or panitumumab. In addition, *BRAF* V600E mutation makes response to panitumumab or cetuximab highly unlikely. Testing for *KRAS*, *NRAS*, and *BRAF* mutations should be performed only in certified laboratories and can be performed on formalin-fixed paraffin-embedded (FFPE) tissue. The testing can be performed on either the primary colorectal cancers or the metastases, as literature has shown that the *KRAS*, *NRAS*, and *BRAF*

mutations are similar in both specimen types (NCCN.org, 2017). Additionally, microsatellite instability (MSI) or mismatch repair (MMR) testing is recommended in all patients with a personal family history of colon or rectal cancer. Microsatellite instability results in an increase in specific types of mutations and is a result of inactivating mutations in any of several genes involved in a particular pathway of DNA mismatch repair. Generally, indication of MSI is evidence that there are impairments in MMR. Treatment options for MMR deficient cancers have included chemotherapy, oxaliplatin, and irinotecan. Immunotherapy (Keytruda) has also been approved for treatment of MMR deficient cancers. The presence of *BRAF* V600E mutation in the setting of *MLH* absence would preclude the diagnosis of Lynch syndrome. Stage II MSI-H patients may have a good prognosis and do not benefit from 5-FU adjuvant therapy (NCCN, 2017). See **Figure 3** for schema of NCCN guidelines for CRC treatment.

NCCN also mentions *ERBB2* (previously known as *HER2*) overexpression in the treatment guidelines. Although *HER2* is rarely overexpressed in CRC, the prevalence is 5 to 14% higher in patients that are *KRAS/BRAF* wild type (NCCN, 2017). Diagnostic methods and therapeutic approaches for *HER2* testing in CRC are still in the investigational phase. However, initial results indicate *HER2* overexpression may be predictive of resistance to *EGFR*-targeting monoclonal antibodies (NCCN, 2017).

Genomic Analysis

As described above, there are a number of anticancer therapeutic agents that work by targeting specific proteins that are expressed by tumors. While challenging to

clinically prove across many cancers, some early studies have shown that selecting therapeutic agents for patients based on the genetic alterations they harbor has increased efficacy (Johnson *et al.,* 2014)). This calls upon the need for comprehensive characterization of cancer genomes and the ability to identify patients who are likely to respond to particular therapies. Technological advances have allowed the field of genomics to evolve from the Sanger method for DNA sequencing to microarrays to massively parallel sequencing (MPS) (MacConaill, 2013). Increased understandings of biological driver events and advances in technology over the past decade have allowed better detection of somatic cancer alterations. This, in effect, has led to advances in the personalized medicine spectrum. For example, in 2010, OncoMap, a high-throughput genotyping platform, was used to characterize the mutation status of a panel of known cancer genes. Using OncoMap, researchers at the Center for Cancer Genome Discovery at Dana-Farber Cancer Institute were able to examine 474 mutations in 41 known cancer genes for gastric carcinomas. They found that *PIK3CA* mutations were the most frequent (Lee *et al.,* 2012). This study also paved the way for some insight into screening for potential druggable targets in gastrointestinal cancers. In 2017, researchers used OncoMap to perform extended RAS mutational analysis on 227 wild type *KRAS* exon 2 CRC patients. They were able to detect 57 additional mutations (11% in *KRAS* exon 2 and 14% beyond exon 2), and effectively select candidates for treatment with cetuximab (Kim *et al.,* 2017). Without the use of a high-throughput extended genotyping approach, this would not be feasible.

Around the time of large-scale genotyping to assay for cancer mutations, technological advances in “next generation” or massively parallel sequencing were

beginning to revolutionize the field of genomics. These technologies have enabled advancements in the comprehensive profiling of tumors by having the ability to interrogate DNA, RNA or chromatin. In comparison to previously available sequencing capabilities (Sanger sequencing), MPS not only reduces the cost of testing, but also improves both the sensitivity and scalability of sequencing (Wagle *et al.,* 2012; Sholl *et al.,* 2016). In addition, one can interrogate varying proportions of the genome, including whole exome, transcriptome, or a subset of genomic regions. DNA-Seq, RNA-Seq, ChIP-Seq and methyl-Seq are all types of massively parallel applications that can be used to obtain information on genetic and gene expression-based alterations (MacConaill, 2013).

Additional improvements in applications of MPS allow researchers to select the specific areas of the genome to be interrogated, thus again reducing cost and improving sensitivity. This can be achieved by hybrid capture or targeted PCR, such as amplicon sequencing. Improvements in throughput of sequencing machines have now enabled researchers to interrogate the entire exome (whole exome sequencing, WES) in one single sequencing lane, for under a thousand dollars (MacConaill, 2013).

Clinical Applications of Genomic Technologies

There are several commercial multigene assays that have been developed to better provide prognostic and predictive information in regards to cancer therapy for patients with stage II or III colon cancer. These include both expression assays and DNA tests. Oncotype DX (Genomic Health) quantifies the expression of recurrence-risk genes. This assay is used to classify the likelihood of recurrence of cancer. Another assay, ColoPrint

(Agendia), quantifies the expression of 18 genes to classify recurrence risk. A DNA test, ColDX (Almac), is a microarray-based multigene assay that uses 634 probes to identify patients with stage II colon cancer with high risk of recurrence. Asides from predicting recurrence, biomarker testing is also essential to identify which therapy is appropriate for the specific patient.

Genomic analysis tests in the clinical setting have evolved from traditional single gene assays to a large degree due to the development of new technologies like genotyping and massively parallel sequencing (MPS). MPS in particular has revolutionized our ability to generate a comprehensive analysis of many genes from small, often FFPE cancer specimens with limited amounts of material. Approaches such as hybrid capture have enabled profiling at a sensitivity, depth and breadth that was previously unfeasible (Wagle *et al.,* 2012). Hybrid capture involves the detection of a DNA target that is hybridized to an RNA molecule.

The Praxis extended RAS Panel by Illumina is a new companion diagnostic tool for use with panitumumab (*EFGR* inhibitor) therapy. It is the first FDA-approved, massively parallel sequencing-based in vitro diagnostic (IVD) for metastatic colorectal cancer. The Praxis extended RAS Panel has the ability to detect 56 RAS mutational variants [*KRAS* (exons 2, 3, and 4) and *NRAS* (exons 2, 3, and 4)] in DNA extracted from FFPE colorectal cancer tissue samples. This gives physicians meaningful insights that can aid in the selection of appropriate therapy. It is intended to be used on the Illumina MiSeqDx instrument. *EGFR* inhibitor therapy is used for patients that are wild type RAS as RAS mutations would confer resistance to anti-*EGFR* therapy and result in activation of the RAS-RAF-MEK-ERK pathway.

Academic Cancer Centers and MPS

Many cancer centers in the US and beyond have developed targeted MPS tests that can be used for research as well as clinical purposes. OncoPanel is a cancer specific platform that utilizes hybrid capture and MPS, and, selectively targets several hundred important cancer genes sequencing used at DFCI (Wagle *et al.,* 2012; Sholl *et al.,* 2016). MSK-IMPACT (Integrated Mutation Profiling of Actionable Cancer Targets), another hybrid capture profiling platform, utilizes a solution-phase exon capture and next generation sequencing to detect somatic genetic alterations in FFPE tumor specimens (Cheng *et al.,* 2017).

PROFILE

The PROFILE research project is one of the nation's most comprehensive personalized cancer therapy projects and is a joint venture taking place at Dana-Farber Cancer Institute (DFCI), Brigham and Women's Hospital (BWH), and Boston Children's Hospital (BCH) in Boston, MA. All patients are offered the opportunity to consent to genomic testing of their tumor. DNA samples are taken from existing tumor tissues (mostly FFPE) from patients' at DFCI, BWH, and BCH. These samples are then analyzed using a targeted MPS assay called OncoPanel to produce a patient specific 'tumor profile.' Every patient has a different pattern of mutations and alterations, which make up that patient's tumor profile. This genomic information can be used clinically to prescribe therapy that is targeted towards the patient's specific tumor profile, making cancer

therapy more effective and more efficient. As of September 2017, over 46,000 patients have consented to be a part of this project and more than 20,000 genetic personal tumor profiles have been completed (http://www.dana-farber.org, 2016). The overall programmatic approach is summarized in **Figure 4**.

There have been three versions of the OncoPanel test. OncoPanel v1 targeted 275 genes covering 1.7Mb of the human genome. OncoPanel v2 targeted 302 genes covering 1.9Mb of the human genome. OncoPanel v3 targets 477 genes covering 3.2Mb of the human genome. In the future, the PROFILE project aims to include germline and other sequencing capabilities, such as RNA sequencing and circulating tumor DNA sequencing, in their analyses (http://www.dana-farber.org, 2016). Researchers have created a massive database of tumor genetic profiling data from this very large cohort of patients. This data can serve as the foundations to many advances in cancer research.

The cBioPortal (http://www.cbioportal.org) for cancer genomics is an open access, open-source resource for interactive exploration of multidimensional caner genomic data sets. This online portal provides visualization, analysis, and download of large-scale data. The type of data available in the public portal includes DNA copy-number data, mRNA and microRNA expression data, non-synonymous mutations, protein-level and phosphorylation level data, DNA methylation data, and limited de-identified clinical data. The publically available data is curated from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov) data center and is regularly updated (Gao *et al.,* 2013; Cerami *et al.,* 2012). Data from the PROFILE project can be accessed and analyzed using a private cBioPortal for DFCI.

Matchminer (https://matchminer.dfci.harvard.edu) is a computational platform used for matching patient-specific genomic profiles to precision cancer medicine trials that are currently available at DFCI. This online portal provides trial search, patient-specific matching, and patient recruitment for specific trials. The genomic data available in the portal is automatically imported from the Profile project, under DFCI protocol 11-104.

Research Aims, Goals, and Hypotheses

Previous studies have shown that 40% of CRCs have *KRAS* mutations, and an additional 10% of CRC patients harbor activating *BRAF* mutations. Thus, about 50% of CRC cases are wild type for *KRAS* and *BRAF* mutations (Morkel *et al.,* 2015). The purpose of this study is to identify key genomic drivers of CRC in cases that are wild type for *KRAS* and *BRAF* mutations. Characterizing the driver events for these cases could lead to insights into recommendations for personalized cancer therapy.

Primary objective: Describe the mutational landscape *KRAS* and *BRAF* wild type colorectal cancer (CRC).

Specific Aim 1: Define two cohorts of CRC patients from PROFILE; one cohort for "wild type" for *KRAS* and *BRAF* mutations, and one cohort for *KRAS* and *BRAS* mutated.

Specific Aim 2: Characterize the genomic landscape of wild type cohort and compare with the mutant cohort.

Specific Aim 3: Identify driver alterations that may be potential targets in wild type cohort, and determine the frequency of these and any correlations with pathological or clinical characteristics such as age and gender.

Chapter II

Materials and Methods

The data used was obtained from the extensive database of tumor genome sequences obtained through the PROFILE research initiative and downloaded from OncDRS (Orechia *et al.,* 2015). DFCI protocols for data usage were strictly adhered. This study focuses on identifying possible driver alterations in *KRAS/BRAF* wild type CRC and determining if any specific alteration is associated with clinicopathologic characteristics. This process includes characterization of the wide spectrum of genomic alterations that are detectable in *KRAS/BRAF* wild type CRC.

Patient Selection:

All patients in this study were consented under Dana-Farber Cancer Institute International Review Board (IRB) protocol 11-104. All patients at DFCI, BWH, and BCH were invited to participate in the study. The patient samples were selected and analyzed in a CLIA-certified laboratory at the Center for Advanced Molecular Diagnostics at BWH. All cancer types, stages, and grades were tested without any restrictions.

OncoPanel:

Samples used for testing included FFPE tissue sections, cytological cellblocks, freshly frozen tissue or cell pellets, fresh peripheral blood or bone marrow, and slides containing smear preparations. Tumor content was estimated by an anatomic pathologist for corresponding slides, cell count, or flow cytometry for fresh blood and bone marrow samples. Tissue sections were manually dissected off corresponding unstained sections or cored directly from the paraffin block. Paired germline samples were not analyzed. DNA was isolated using a commercially available kit (Qiagen, Valencia, CA) and quantified (PicoGreen, ThermoFisher Scientific, Waltham, MA).

The procedure to prepare hybrid capture libraries has been previously described (Sholl *et al.,* 2016). As such, DNA libraries were hybridized to a set of custom probes targeting the appropriate set of genes (depending on the version of OncoPanel). Sequencing was performed using Illumina HiSeq 2500 with 2x100 paired-end reads to a mean target coverage of 187X unique, high quality, mapped reads per sample. Specimens were genotyped across 48 single nucleotide polymorphisms (SNPs) selected for 45-55% heterozygosity. This was to confirm patient identity and avoid mix up between the samples. SNP identity at the targeted sites was resolved by single base extension and analysis by MALDI-TOF mass spectrometry using the MassArray System (Agena Bioscience). Genotyping results were correlated with results obtained during OncoPanel sequencing, with 80% concordance. (Sholl *et al.,* 2016).

Data Analysis:

Data used for this study was analyzed with an internally developed bioinformatics pipeline. This consists of publically available tools and internally developed algorithms, including VisCap Cancer (Pugh *et al.,* 2016), Phaser, BreaKmer (Abo *et al.,* 2015), and Picard (http://broadinstitute.github.io/picard/). Picard was used for pooled sample reads. GATK (https://software.broadinstitute.org/gatk/) was used to refine alignments around insertion/deletion (Indels) sites. Single nucleotide variants (SNVs) were called using MuTect (Cibulskis et al., 2013) and indels using Indelocator (http://archive.broadinstitute.org/cancer/cga/indelocator). Oncotator (http://portals.broadinstitute.org/oncotator/) was used for annotation.

Tumor mutational burden (TMB) was calculated by determining the number of non-synonymous somatic mutations that occur per megabase of exonic sequence data across all genes on the pane. Using the mutational burden calculated in this manner as a proxy for neoantigen load has been described in several papers (Rizvi *et al.,* 2015; Snyder *et al.,* 2014; Roszik *et al.,* 2016).

Multiple measures of DNA mutational patterns were calculated for each tumor, including the number of small insertion/deletion events that occur in homopolymer regions per megabase of exonic sequence data and the overall mutational burden per megabase (Nowak *et al.,* 2017). A threshold of >1.5 insertions/deletions in homopolymer regions per megabase optimally classified cases as mismatch-repair deficient (MMR-D), yielding a sensitivity of 97.9% and specificity of 98%.

Genomic data was provided using The Oncology Data Retrieval System (OncDRS) for DFCI. OncDRS is a self-service application for investigators, which

allows data query, data request, data access, and data extraction. Additional data for these cases was also downloaded using the private cBioPortal interface for DFCI, which provides visualization, analysis, and download of large-scale cancer genomic data sets (Gao *et al.,* 2013; Cerami *et al.,* 2012). All of the data was consolidated and sorted into two main cohorts; *KRAS/BRAF* mutant and *KRAS/BRAF* wild type. The genomic data was analyzed using various genetic analysis tools and databases available online, including Microsoft Excel, R for bioinformatics, cBioPortal, and GraphPad Prism.

Matchminer (https://matchminer.dfci.harvard.edu) was used to obtain a list of currently available clinical trials and the genomic targets associated. This data was used to identify potential candidates for clinical trials from our cohort of CRC patients.

Statistical Analysis:

Categorical comparisons were performed using Fisher's exact or Chi square tests with Bonferroni correction for multiple comparisons. Sample means were compared using a 2-tailed Student's t test assuming equal variance. Correlation of mutational frequency with clinicopathologic features assessed using Pearson correlation coefficient. P values of <0.05 were considered significant.

Chapter III

Results

Characteristics of the Cohort

There were 1063 total CRC cases used in this analysis. Cases that harbored any mutation in *KRAS*, *BRAF*, or both were categorized into the *KRAS*/*BRAF* mutant cohort. For the purpose of this study, all mutations in *KRAS* and *BRAF* were used, instead of only known activating mutations such as *KRAS* exon 2 and *BRAF* V600E. A total of 571 (53.7%) cases fell into the *KRAS/BRAF* mutant category (**Figure 5**). 95% of *KRAS* mutations were known activating mutations, such as G12A and G13D (**Figure 6**). 63% of total *BRAF* mutations were the canonical activating V600E mutation (**Figure 7**). The remaining 492 (46.3%) cases did not show any *KRAS* or *BRAF* mutation and were categorized into the *KRAS/BRAF* wild type cohort. See **Table 3** for a description of the cohort.

There were 274 (48%) males and 297 (52%) females in the mutant cohort. The wild type cohort had 292 (59%) males and 200 (41%) females. There was no significant difference in the number of males and females in both cohorts. The median age for the mutant cohort was 56 years, with a range of 19 years to 90 years. The median age of the wild type cohort was 57 years, with a range of 13 years to 87 years. The difference in median age for both cohorts was not significant.

The detailed cancer type was also noted for each cohort. The majority of cases (>98%) presented with colon/rectal or colorectal adenocarcinoma as the primary

diagnosis. A few cases presented with other cancer types due to metastasis. The primary cancer diagnosis for these patients was CRC, however these patients had metastatic recurrences in other sites, such as breast and esophagus. Such patients were included in the analysis as their primary diagnosis was CRC. **Table 4** outlines the detailed cancer types in the mutant and wild type cohorts.

Frequency of Mutations

Description of the overall cohort

The top 25 gene mutations were calculated for each cohort (**Table 5**). Interestingly, there were some mutations that occurred more frequently in one cohort than the other. Mutations in *APC*, *TP53*, and *PIK3CA* were the top 3 genes in both cohorts. There was no statistically significant difference observed for *APC* mutations (424/571 (74%) in the mutant cohort and 397/492 (81%) in the wild type cohort, p-value 0.23), however, mutations in *TP53* (352/571 (62%) in the mutant cohort and 379/492 (77%) in the wild type cohort) were more common in the wild type (p-value 0.0025). Mutations in *PIK3CA* (156/571 (27%) in the mutant cohort and 77/492 (16%) in the wild type cohort) were significantly higher in the mutant versus wild type cohort (p-value 5.07×10^{-05}).

Other gene mutations that appeared in the top 25 of both cohorts included *KMT2D*, *SOX9*, *ARID1A*, *FBXW7*, *SMD4*, *ATM*, *BRCA2*, *TCF7L2*, *ARID1B*, *NOTCH1*, *PRKDC*, and *CREBBP*. **Table 6** shows all of the respective comparisons and p-values. A p-value of <0.05 was considered to be statistically significant.

Mutations in *ATM* (110/571 (19%) mutant and. 46/492 (8%) wild type, p-value 2.58x10-5), *SOX9* (100/571 (18%) mutant and 53/492 (11%) wild type, p-value 0.003),

SMAD4 (85/571 (15%) mutant and 53/492 (11%) wild type, p-value 0.01), *FBXW7* (88/571 (15%) mutant and 50/492 (10%) wild type, p-value 0.02), and *PRKDC* (78/571 (14%) mutant and 42/492 (9%) wild type, p-value 0.01) were more frequent in the mutant cohort. However, *NRAS* mutations were more frequent in the wild type cohort (48/492 (9.8%)) compared to the mutant cohort (13/571 (2.3%) with a p-value of (3x10-7).

The difference in mutational frequency between the two groups for clinically actionable genes (have targeted inhibitors or clinical trials available) (**Table 7**) was calculated using Chi Squared Test. These include *AKT1, AKT2, AKT3, ALK, ATM, BRAF, BRCA1, EGFR, ERBB2, FLT3, HRAS, IDH1, MAP2K1, MTOR, NRAS, TSC1*, and *TSC2*. There were significantly more mutations in *AKT1, AKT2, AKT3, ALK, ATM*, and *MTOR* in the mutant cohort. As previously described, the wild type cohort had a higher frequency of *NRAS* mutations.

Mutational frequency for genes in both cohorts was compared for genes that are druggable targets in currently available clinical trials at DFCI. This includes *ALK, JAK1, JAK2, MYCN, NOTCH1, NOTCH2, NOTCH3, NTRK1, NTRK2, NTRK3, ROS1* and *RSPO* in addition to the actionable genes already mentioned. See **Table 8** for a list of clinical trials currently available and the gene targets for those trials. The mutational frequency was not significantly higher in the wild type cohort compared to the mutant cohort for any of the above mentioned genes. However, there were significantly more mutations in the mutant cohort for the following genes: for *ALK* (53/571 (9.2%) mutant and 24/492 (4.8%) wild type, p-value 0.007), *NOTCH2* (39/571(6.7%) mutant and 17/492 (3.4%) wild type, p-value 0.016), *NOTCH3* (29/571 (5%) mutant and 13/492 (2.6%) wild

type, p-value 0.046), *NTRK1* (25/571 (4.3%) mutant and 8/492 (1.6%) wild type, p-value 0.011), and *ROS1* (60/571 (10.3%) mutant and 22/492 (4.4%) wild type, p-value 0.0004).

There was no significant difference in the frequency of *EGFR* mutations in both wild type and mutant cohorts (p-value 0.29). There were 19/492 (3.8%) cases with *EGFR* mutations in the wild type cohort and 30/571(5.2%) cases with *EGFR* mutations in the mutant cohort. In addition, the overall spectrum of mutations for both cohorts was relatively similar, with no major differences noted (**Figure 8**).

The difference in frequency of *ERBB2* (38/571 (6.6%) mutant and 29/492 (5.8%) wild type), *TSC1* (28/571 (4.9%) mutant and. 13/492 (2.6%) wild type), *TSC2* (38/571 (6.6%) mutant and 30/492 (6%) wild type), and *MTOR* (53/571 (9.2%) mutant and 21/492 (4.2%) wild type) between the two cohorts was not significant. Of the 29 cases with *ERBB2* mutations in the wild type cohort, 28 of the mutations were missense and 1 was a splice region mutation which co-occurred with an *ERBB2* missense mutation in the same patient (**Figure 9**).

NRAS

Mutations in *NRAS* (13/571 (2%) mutant and. 48/492 (9.7%) wild type) were more frequent in the wild type cohort (p-value 3.86x10-7). Because there were significantly more *NRAS* mutations in the WT cohort, the next step was to assess if these are activating mutations and what other mutations they are co-occurring with. All of the *NRAS* mutations in the WT cohort were recurrent hotspot mutations, such as G12A, G12C, G13D, and A146T. These are all known oncogenic driving mutations (See **Figure 10**). It was noted that *NRAS* mutations in the wild type cohort frequently co-occurred

with *FAT1, ERCC4, ARID2, SQSTM1, BUB1B, PTPN14*, and *RUNX1T1*. In addition, *NRAS* mutations in the wild type cohort displayed mutual exclusivity with *ERBB2* (p-value 0.04).

The mutant cohort consisted of all patients that have mutations in *KRAS* or *BRAF*. 16 cases (2.8%) in this cohort harbored concomitant *KRAS* and *BRAF* mutations, which accounts for 1.7% of the overall CRC cohort (16/1063 patients). There is evidence to suggest that co-existing *BRAF* and *KRAS* mutations may indicate poor prognosis (Vittal, Midditi, & Kumar, 2017). There was no significant difference in the number of males and females in this group.

Similarly, there were 13 *NRAS* mutations in the mutant cohort. Eight of these were activating *NRAS* mutations and the remaining may just be passenger mutations. *NRAS* mutations in the mutant cohort were not expected because *NRAS* is typically mutually exclusive with *KRAS* mutations. There were 3 cases with co-occurring *NRAS* and *KRAS* mutations and 5 cases with co-occurring *NRAS* and *BRAF* mutations. There was no significant difference in the number of males and females in this group.

The Cancer Genome Atlas (TCGA) and COSMIC Data

In order to compare the results from the CRC cohort used for this study, other published and publically available data was also analyzed as a point of reference. CRC data available through TCGA (http://cancergenome.nih.gov/.) and The Catalog of Somatic Mutations in Cancer (COSMIC) (cancer.sanger.ac.uk) was used for this purpose. The data from both TCGA and Sanger COSMIC for CRC had similar mutational patterns as seen in the cohort used for this study. The most commonly mutated genes reported include *APC, TP53, KRAS*, and *PIK3CA* among others. As expected, there were

approximately 50% of cases wild type for *KRAS* and *BRAF* mutations, with 36% *KRAS* and 9% *BRAF* mutations present. Overall, the distribution of mutations across our cohort is as expected with respect to the CRC mutational data available from TCGA and COSMIC. See **Figure 11** for distribution of most frequently mutated genes for TCGA and COSMIC data.

Copy Number Analysis

Copy number analysis was performed for cases that had copy number data available. From the overall CRC cohort (1063 patients) copy number data was available for 798 patients (362 in the wild type cohort and 436 in the mutant cohort). There were 14 cases (1.8% of overall CRC cohort) cases with *ERBB2* amplifications. Of these, 12 cases (3% of wild type cohort) were in the wild type cohort and 2 cases (0.5% of mutant cohort) in the mutant cohort, p-value 0.002. There were 132 low copy gains of *ERBB2*, forty-one (11% of wild type cohort) in the wild type and ninety-one (20% of mutant cohort) in the mutant cohort, p-value 0.0009. There were 7 amplifications in *EGFR* (0.9% of overall CRC cohort), and all were in the wild type cohort, p-value 0.003. Similarly, there were 6 amplifications observed in *FGFR1* (0.75% of overall CRC cohort) and all were in the wild type cohort, p-value 0.007.

There was no significant difference in the number of amplifications in *CCNDI*, *CCND2*, *CCND3*, *CCNE1* and *MYC* between the two cohorts. There were 6 amplifications in *CCND1*, of which 4 were in the wild type cohort, p-value 0.29. There were 10 amplifications in *CCND2*, of which 5 cases (1.4% of wild type cohort) were in

the wild type cohort, p-value 0.76. There were 2 amplifications in *CCND3* and were only seen in the mutant cohort. Of the 3 amplifications in *CCNE1*, only one was in the wild type cohort. There was only one patient with a *CDK6* amplification, and this patient was in the wild type cohort. There were 29 amplifications in *MYC* (12 (3.3%) in the wild type cohort and 17 (3.9%) in the mutant cohort, p-value 0.6).

The frequency and distribution of genes with single (1D) and two copy losses (2DEL) were also analyzed. There were 17 cases (3.9%) in the wild type cohort with *PTEN* mutation with 1D single copy deletion, as compared to 62 cases (12.2%) in the mutant cohort, p-value 5x10-5. There were 12 two copy loss in *PTEN* (10 (2.7%) in the wild type cohort and 2 (0.45%) in the mutant cohort), p-value 0.0.008. Although there were significantly more *PTEN* single copy losses in the mutant cohort, there were significantly more *PTEN* double deletions in the wild type cohort. Integrative analysis was done for the cases with single copy loss plus mutation in *PTEN*. Such patients were counted as 2 copy loss. This observation increased the number of deletions in both cohorts to (64 cases) in the mutant cohort and (27 cases) in the wild type cohort, p-value 0.002. *PTEN* is a tumor suppressor gene and plays an important role in the development of CRC. Loss of *PTEN* function increases genomic instability.

There was no significant difference in mutant and wild type cohorts for deletions in *MET*, *CDKN2A*, *CDKN2B*, *SMAD2*, *SMAD4*, or *TP53*. There were 9 single deletions in *MET*, 5 cases (1.4%) in the wild type cohort and 4 cases (0.9%) in the mutant cohort. There were 10 cases with 2DEL (6 cases (1.7%) in the wild type cohort and 4 cases (0.9%) in the mutant cohort) in *CDKN2A* and *CDKN2B*. There were 7 cases with 2DEL in *SMAD2* (3 cases (0.8%) in the wild type cohort and 4 cases (0.9%) in the mutant

cohort). There were 16 cases with 2DEL in *SMAD4* (4 (1.1%) in the wild type cohort and 12 cases (2.75%) in the mutant cohort). *TP53* is also a tumor suppressor gene. Three 2DEL were seen in *TP53* with 1 case (0.3%) in wild type cohort and 2 cases (0.45%) in the mutant cohort. See **Table 9** for a list of observed copy number variations in both cohorts.

Structural Variant Analysis

Using the BreaKmer algorithm (Abo *et al.,* 2015), 259 patients (139 (54%) mutant and 120 (46%) wild type) with structural rearrangements were identified. The most commonly recurring structural variants were identified in *APC* (7 cases (5%) in the mutant cohort, 6 cases (5%) in the wild type cohort), *CTNNB1* (8 cases (6%) in the mutant cohort, 9 cases (8%) in the wild type cohort), and *TP53* (4 cases (3%) in the mutant cohort, 10 cases (8%) in the wild type cohort). There was 1 case in the wild type cohort with a rearrangement involving *AKT2* and *RGS7*. There was also one *AKT3* intragenic rearrangement in the wild type cohort. The mutant cohort had 1 patient with *AKT1* intragenic rearrangement.

Structural variant review also identified a 369 base pair deletion in *TSC2* intron 7 extending to V221 in exon 8. This is likely to lead to loss of function of *TSC2*. One case with *MTOR* mutation had identified a rearrangement between exon 30 of *MTOR* and chromosome 12q21.33. There were 2 cases in the wild type cohort with *BRCA1* structural rearrangements. There was also one case with a *BRCA2* rearrangement. There were no structural variants for *BRCA1* or *BRCA2* detected in the mutant cohort.

ALK fusion rearrangements are potential drug targets, thus, it is important to look for these in the cohorts. Structural variant analysis, using the BreaKmer algorithm, identified a single case with *CAD-ALK* rearrangement. This patient is a 54-year-old female from the WT cohort. This patient also has mutations in *TP53*, *ARID1B*, *IGF1R*, *CHEK2*, *MAP2K1*, *CADM2*, and *CDK5*. This is interesting since there are no FDA approved therapies specific to any of these mutations, but there is an approved therapy that can be used with *ALK* rearrangements.

Mismatch Repair and Tumor Mutational Burden

Mismatch repair (MMR) status was obtained for cases in both wild type and mutant cohorts. Analysis was run on a total 339 cases (113 cases from the wild type cohort and 226 cases from the mutant cohort). MMR status was deficient for 29 (12.8%) cases in the mutant cohort versus only 4 cases (3.5%) in the wild type cohort. There were significantly more MMR deficient cases in the mutant cohort (p value 0.009). MMR status was proficient in 197 (87%) cases in the mutant cohort and 109 (96%) cases in the wild type cohort. There were a total 66/492 (13%) of cases with mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2* in the wild type cohort, and 88/571 (15%) in the mutant cohort. There were 21/571 (4%) *POLE* mutations in the mutant cohort, and 9/492 (1.8%) in the wild type cohort. The difference in frequency for *POLE* mutations was not significant (p-value 0.07).

The median Tumor Mutational Burden (TMB) in the mutant cohort was 9.45 mutations per Megabase (range 1.18 to 322.41) and 7.96 mutations per Megabase (range

1.18 to 148.76) in the wild type cohort. The difference in the median of both cohorts was not statistically significant. High TMB has been described as >20 mutations per Megabase (Goodman *et at.,* 2017). As such, >20 mutations per Megabase was used as a cutoff for high tumor mutational burden. TMB was significantly higher in the mutant cohort as compared to the wild type cohort. There were 5.4% of patients with high TMB in the wild type cohort, compared to 16.6% from the mutant cohort, p-value $7.3x10^{-8}$.

Alterations in Actionable Pathways

Targeting gene level mutations is often the common approach, but genes are part of larger pathways and analyzing genes associated with the same molecular pathway may pave way into new insights for patient testing and selection into different treatment approaches. Numbers may not be significant at the gene level, but hold the possibility of significance when analyzed at the pathway level. For this study, three actionable molecular pathways (RAS/RAF/MEK/ERK, PIK3CA/AKT/MTOR, and RTK (receptor tyrosine kinase) and the associated genes were assessed. Actionable pathways are described as molecular or cellular pathways that either already have established targeted therapies to combat mutations occurring in these pathways or are potential targets for drug development. See **Table 10** for a list of genes and associated pathways.

Only 25% (121 of 492) of patients in the wild type cohort had mutations in the RAS/RAF/MEK/ERK pathway, compared to all of the patients (571) in the mutant cohort. There were 51% (294 of 571) patients in the mutant cohort and 34% (169 of 492) patients in the wild type cohort with mutations in the PIK3CA/AKT/MTOR pathway.

58% (32 of 571) of patients in the mutant cohort had mutations in the RTK pathway. 60% (295 of 492) of patients in the wild type cohort had mutations in the RTK pathway. See **Figures 12 - 14**. Comparing the two cohorts, there were significantly more mutations in the RAS/RAF/MEK/ERK pathway (p-value $3x10^{-4}$) and the PIK3CA/AKT/MTOR pathway (p-value $5x10^{-5}$) in the mutant cohort. There was no significant difference in the number of mutations in the RTK pathway between both cohorts.

Candidates for Clinical Trials

There are 62 currently available clinical trials for inhibitors that target specific genes for colorectal cancer and all solid tumors at DFCI. See **Table 8** for a list of trials and the corresponding gene targets, retrieved from Matchminer (https://matchminer.dfci.harvard.edu). Gene mutational frequency, copy number genes, structural variants, mismatch repair status, and tumor types were used to assess potential candidates for current trials in both mutant and wild type groups. There are several trials that target wild type *KRAS*. For these trials, all of the wild type cohort would qualify (492 cases). In addition, there were several trials that target mutant *KRAS* and *BRAF*. For these trials, all of the mutant cohort would qualify (571 cases). Among these, there were two trials that target mutant *KRAS*, specifically p.G12, p.G13, and p.Q61. There were 28% patients in the mutant cohort that qualify for these trials. Gene targets corresponding with available trials included mutations in *AKT1, BRAF, DDR2, EGFR, ERBB2, FGFR1, FGFR2, FGFR3, GNA11, GNAQ, IDH1, KRAS, MET, NRAS, PIK3CA, PTEN, RET*, and *SMO*. There were 237 (48%) patients in the wild type cohort that harbor mutations in the

above genes. After adjusting for *KRAS* and *BRAF* mutations, there were 291 (51%) patients in the mutant cohort that harbor mutations in the above genes.

Certain trials have copy number variants as targets. There were 112 (25%) patients in the mutant cohort with targeted copy number variations. There were 91 (25%) patients in the wild type cohort with targeted copy number variations. There were 3 patients in the mutant cohort that had targeted structural variants, and 4 cases in the wild type cohort with targeted structural variants who qualify for trials targeting *ALK, BRAF, FLT3, MTOR, ROS1* and *TSC2*. There are two available trials that target MMR-deficient. There were 4 (12% of all MMR-deficient) cases in the wild type that qualify for these trials. Additionally, 252 (51%) of wild type cases qualify for solid tumor trials. In total, considering targeted gene mutations, copy number variations, structural variations, and MMR status, there were 351 patients in the wild type cohort (71%) that would qualify for a currently available clinical trial at DFCI.

Other Pathology and Clinical Variables

Tumor staging and laterality information was only available for 444 patients (253 cases (57%) in the mutant cohort and 191 cases (43%) in the wild type cohort). CRC was left sided for 6 patients (2 patients (0.5%) in the mutant cohort and 4 patients (0.9%) in the wild type cohort). CRC was right sided for 4 patients (3 (1.2%) in the mutant cohort and 1 patient (0.5%) in the wild type cohort). This difference was not statistically significant.

An interesting find for the wild type cohort was the presence of 12 patients with amplifications in *ERBB2*, which was significantly higher than the 2 found in the mutant cohort (p-value 0.002). For further analysis, other pathology and clinical variables were analyzed for these cases. This included gender, age, primary cancer diagnosis, left or right CRC, stage, and grade. All of the patients with *ERBB2* amplifications at 17q12 also had amplifications in *IKZF3* at 17q21. Additionally, all of these patients had mutations in tumor suppressor genes, *TP53* and *APC*. One patient also had a mutation in *BRCA2*. The range of ages for these 12 patients was 27 years to 81 years, with a median age of 56 years. There were 7 males and 5 females. There were 7 patients with CRC not on a paired site, and laterality information was not available for the remaining patients. Two patients had stage 3 cancer and four patients had stage 4 cancer. Staging information was not available for the remaining patients. See **Table 11** for detailed clinicopathologic features for the patients with *ERBB2* amplifications. Frequently occurring mutations in the 12 patients with *ERBB2* amplifications include *TP53*, *APC*, *REL*, and *STAG1* (See **Table 14** for a list of genes mutated in cases with *ERBB2* amplifications. See **Figure 15** for a comparison of commonly mutated genes in *ERBB2* amplified, the wild type cohort, and the mutant cohort).

Additionally, the wild type cohort had 7 patients with amplifications in *EGFR*. All of these patients were in the wild type cohort, p-value 0.003. There were 3 cases with metastatic recurrences, and the remaining cases had primary CRC. Tumor laterality and stage information was not available for these cases. The median age for patients with *EGFR* amplifications was 46 years, with a range of 31 to 85 years, which significantly

different from the median age of the overall CRC cohort (56 years) (p value 0.006). See **Table 12** for clinicopathologic features of patients with *EGFR* amplifications.

Similarly, there were 6 patients with high amplifications in *FGFR1*, compared to 0 cases found in the mutant cohort, p-value 0.01. Tumor laterality and stage information was not available for these cases. There were 2 metastatic recurrences and the remaining cases had CRC primary cancer diagnosis. The median age for patients with *FGFR1* amplifications was all of high amplification cases was 46 years, with a range of 39 to 58. See **Table 13** for clinicopathologic features of patients with *FGFR1* amplifications.

Chapter IV

Discussion

Significance of Results

Description of the Cohort

The overall colorectal cancer cohort in this study consisted of 1063 patients, of which 46% were wild type for *KRAS* and *BRAF* mutations. Over 50% of the patients had *KRAS* and/or *BRAF* mutations acting as the oncogenic drivers of cancer, however, the mutational landscape of *KRAS*/*BRAF* wild type CRC remained to be described. Consistent with previously reported colorectal cancer cases, made publically available through The Cancer Genome Atlas, the most common gene mutations in the overall cohort occurred in *APC, TP53, KRAS, PIK3CA, FBXW7, SMAD4*, and *TCF7L2*. There was no significant difference between the gender and range of ages between the mutant and wild type cohort. The median age at sequencing for both cohorts was 56, with a range of 13 to 90 years. The NCCN estimates an increase by 90% for colon cancer and 124.2% for rectal cancer in patients younger than 50 years by the year 2030 (NCCN, 2017). With a median age of 56, it is possible that many patients from this study could have been diagnosed at an earlier age (<50 years). This may indicate the need for earlier cancer screening by routine tests and colonoscopy, especially for at-risk populations.

Increased *NRAS* Mutations

NRAS, a GTPase, is mutated in a diverse range of cancers, most frequently in melanoma and thyroid cancer. In this study, it was observed in a higher frequency (9.7%) in the wild type cohort than in the mutant cohort (2%) (p-value $3.86x10^{-07}$). The *NRAS* Q61H, Q61K, Q61L, Q61R, G12C, G12D, G13C, G13D, and G13R mutations are known to be oncogenic, this includes 83% of *NRAS* mutations observed in the wild type cohort. As with all RAS mutations, this mutation represents a contraindication for the use of anti-*EGFR* agents such as cetuximab or panitumumab in patients with colorectal cancer. Not only do patients with tumors harboring this mutation not benefit from treatment with these agents, but some trials have shown a slightly worse outcome for patients with RAS-mutated tumors receiving these agents as compared to those not receiving them.

Although *NRAS* is known to be mutually exclusive to *KRAS* mutations, there were 13 patients with *NRAS* mutations in the mutant cohort. Only 8 of these patients had activating mutations in *NRAS*. Five patients had co-occurring *BRAF* mutations, and three patients had co-occurring *KRAS* mutations. In a pan gene approach, it is usually considered that *KRAS* and *NRAS* are mutually exclusive, however, this study identified cases in which this is not true, and thus, testing for *NRAS* may hold therapeutic insights for patients with *BRAF* or *KRAS* mutated CRC who otherwise may not be have *NRAS* status assessed.

Increased *TP53* Mutations

TP53 is a tumor suppressor gene found in multiple cancer types. The presence of this mutation often indicates a poorer prognosis. CRC has been described using three distinct pathways of genomic instability; chromosomal instability, microsatellite

instability, and CpG Island Mthylator Phenotype (Armaghany *et al.,* 2012). The chromosomal instability (CIN) pathway is associated with a loss of function of tumor suppressor genes *TP53* and *APC*. Mutations in *TP53* were more common in cases wild type for *KRAS* and *BRAF* mutations (77%) versus *KRAS/BRAF* mutant cases (62%), with a p-value of 0.0025. The increased prevalence of *TP53* in the absence of *KRAS* and *BRAF* mutations might indicate that the wild type cohort has more of the chromosomal instability subtype.

66% of the *TP53* mutations in the wild type cohort were missense, with most common protein changes at R273C/H/L, R175C/H, and R248Q/W (**Figure 16**). These mutated amino acids have been identified as recurrent hotspots (statistically significant) in a population-scale cohort of tumor samples of various cancer types (Chang *et al.,* 2016; Gao *et al.,* 2017).

TP53 mutations in the wild type cohort displayed mutual exclusivity patterns to mutations in *ATM, SMAD2, PTEN, SOX9*, and *ERBB2*. In addition, these mutations often co-occurred with *APC, NOTCH3, MAP2K1*, and *MAP2K4* mutations. It was noted that alterations in tumor suppressor genes were more frequent in the wild type cohort compared to the mutant. Although there are currently no FDA-approved or NCCN-compendium listed treatments specifically for patients with *TP53* mutated CRC, the higher incidence of tumor suppressor gene changes in wild type cases (76% wild type, 67% mutant, p value 0.002) may prompt the need for further research on the development of drugs that target tumor suppressors, possibly by gene therapy with viral administration to reactivate wild type *TP53* (Guo *et al.,* 2014).

Increased frequency of *PIK3CA*

There was a significantly higher frequency in mutations in *PIK3CA* in the mutant cohort (27%) compared to the wild type cohort (16%) with a p-value $5.07x10^{-7}$. *PIK3CA* is the catalytic subunit of PI3-kinase and is frequently mutated in a diverse range of cancers. These include breast, endometrial and cervical cancers. *PIK3CA* mutations also commonly occur in colorectal cancer and co-occur with RAS mutation in about two-thirds of cases (Gao *et al.,* 2013). Consistent with observation, *PIK3CA* mutations in this study were seen in a higher frequency among patients with *KRAS/BRAF* mutated CRC. There is currently no FDA-approved or NCCN-compendium listed treatments for patients with *PIK3CA* mutations, however, there are inhibitors, such as GDC-0032 that target copy number variations for *PIK3CA* currently under investigation in clinical trials (Matchminer, 2017). In addition, there are also trials that target wild type *PIK3CA* cases. It may also be beneficial to target other downstream mutations for patients with *PIK3CA* mutations since this mutation plays a major role in the PIK3CA/AKT/ MTOR pathway. In the wild type cohort, there were 169 (34%) patients, compared to 294 (51%) in the mutant cohort, in this pathway (p-value $5x10^{-5}$), which may be clinically important, as there are inhibitors available for genes in this pathway including *AKT1, AKT2, AKT3*, *TSC1*, and *TSC2*.

Other Actionable Genes

The *ATM* gene encodes a tumor suppressor involved in DNA damage repair. Germline mutations of *ATM* are associated with Ataxia talangiectasia and predispose to various cancers, including colorectal cancer. There was a lower frequency of *ATM* mutations in the wild type cohort compared to the mutant. *ATM* mutations often result in

increased cancer predisposition. However, the absence of *ATM*, or *ATM* inhibition, may be a potential mechanism for synthetic lethality in certain cancers (Choi, Kipps & Kurzrock, 2016). Because the wild type cohort had fewer mutations in *ATM* (9%) compared to (19%) in the mutant cohort, it would be interesting to see if any of these cases are *ATM* deficient as a further study. The role of PARP inhibitors could also be evaluated for *ATM* deficient tumors since PARP inhibitors work to inhibit endogenously arising DNA damage.

Mutations in *SOX9, SMAD4, FBXW7*, and *PRKDC* were also seen less frequently in the wild type cohort. *SMAD4* is another tumor suppressor downstream in the TGF- ß signal transduction pathway. It is frequently mutated in CRC and pancreatic cancer, but not as frequent in other cancer types. *SMAD4* mutations were significantly more common in the mutant cohort (p-value 0.01). However, there are currently no FDA approved or NCCN-compendium listed treatments for patients with mutations in S*MAD4, SOX9, FBXW7*, and *PRKDC* in CRC.

Although there was not a significant difference in the mutational frequencies of *TSC1, TSC2*, and *MTOR* in the mutant versus wild type cohorts, it is important to note these mutations as druggable targets. Considering mutations in *TSC1, TSC2*, and *MTOR*, 12.9% cases in the wild type and 20.7% cases in the mutant cohort would be eligible for treatment. *TSC1* and *TSC2* mutations are commonly seen in hepatocellular carcinoma (HCC) and are negative regulators of mechanistic target of rapamycin (*MTOR*) signaling. Loss of function in these genes may be due to deletions. *MTOR* inhibitors, such as everolimus, can be used in patients with inactivating *TSC1* or *TSC2* mutations or activating *MTOR* mutations.

Truncating mutations in *RNF43* have been recently described in CRC cases that show a mutual exclusivity pattern with inactivating *APC* mutations (Giannakis *et al.,* 2014). *RNF43* is a gene that negatively regulates WNT signaling. Mutations in this gene can activate the WNT pathway. As such, WNT secretion inhibitors may be effective for patients with this mutation (Wang *et al.,* 2016). Previous studies have indicated that *RNF43* often co-occurs with *BRAF* V600E mutations. There were 11% of patients in the mutant cohort that has co-occurring *BRAF* and *RNF43* mutations, for which a combinatorial therapy approach may be warranted. In this study, the difference in mutational frequency between the mutant (5%) and wild type (1%) cohorts was not significant.

ERBB2 Mutations

ERBB2 is a receptor tyrosine kinase and is altered by amplification and/or overexpression in various cancers. It is most frequently mutated in breast, esophagogastric and endometrial cancers. There is promising clinical data supporting the use of neratinib in patients with breast cancer harboring oncogenic *ERBB2* alterations, suggesting that neratinib may also be efficacious in patients harboring *ERBB2* mutations in CRC. 5.8% patients had *ERBB2* mutations in wild type cohort compared to 10.02% in the mutant cohort, p-value 0.01 *ERBB2* mutations in the *KRAS/BRAF* wild type cohort included 38% known oncogenic mutations, such as S653Y, R678Q, and V842I (**Figure 9).**

ERBB2 Amplifications

ERBB2 has recently emerged as a new therapeutic target for CRC. Mutations and amplifications in *ERBB2* are present in 7% colorectal cancer cases (Pectasides & Bass, 2015). *ERBB2* amplification is known to be oncogenic and *ERBB2* amplified breast and gastric cancers can be treated by an FDA-approved, *HER2* antagonist, trastuzumab. However, this therapy has not been seen as effective in cases of *ERBB2* amplified CRC. A study has shown efficacy of a combination approach using trastuzumab and lapatinib or pertuzumab with *ERBB2* amplifications in *KRAS* wild type CRC (Sartore-Bianci et al., 2016). *ERBB2* amplified CRC has also shown resistance to *EGFR* antibodies, cetuximab and panitumumab. From the wild type cohort, there were 12 (2.4%) cases with *ERBB2* high amplifications, compared to only 2 (0.4%) in the mutant cohort (**Table 14**). *ERBB2* mutations and amplifications were present in 8.3% of the wild type cohort and 7% in the mutant cohort, p-value 0.4. These patients should be tested for targeted therapy.

CDKN2A Homozygous Deletions

Deletions in tumor suppressor genes are among the most complex type of structural variant identified using current technologies (Macintyre, Ylstra & Brenton, 2016). *CDKN2A* is a tumor suppressor that regulates the cell cycle and is altered by mutation, deletion or epigenetic silencing in a diverse range of cancers, such as melanoma. It is often inactivated by homozygous deletions, resulting cancer predisposition. There were 10 patients with homozygous deletions (6 in the wild type cohort and 4 in the mutant cohort) in *CDKN2A* and *CDKN2B*. The 4 patients with *RAS/RAF* mutant cancers may benefit from CDK inhibitors, Ribociclib and Palbociclib

(Sherr, Beach, & Shapiro, 2015), however, these may not be effective for the wild type cases.

PTEN Deletions

PTEN is a tumor suppressor gene that also plays an important role in the development of CRC. The lipid phosphatase activity of *PTEN* antagonizes the PIK3/AKT/MTOR pathway and thus represses tumor cell growth and survival. Loss of *PTEN* function increases genomic instability by accumulation of PIP3 at the plasma membrane. This activates the PIK3/AKT/MTOR pathway and drives cell growth, proliferation, and survival (Dillon & Miller 2014). Therapeutics that target genes in this pathway, such as Temsirolimus, Everolimus, and Afinitor, may be efficacious for patients harboring *PTEN* deletions. There were 27 cases in the wild type cohort with *PTEN* deletions, as compared to 64 cases in the mutant cohort. This suggests that 11.4% of all CRC cases from this study (3.4% in the wild type cohort) may benefit from screening and therapeutics.

Other Copy Number Differences

CCND1, 2, and *3* are genes that encode proteins that belong to the cyclin family, which function as regulators of CDK kinases. As such, amplification and overexpression of these genes alters cell cycle progression and may contribute to tumorigenesis. As previously described, patients with these mutations may also benefit from CDK inhibitors, Ribociclib and Palbociclib. From the wild type cohort, there were 4 amplifications in *CCND1* and 5 amplifications in *CCND2*. In total, 9 patients from the

wild type cohort (1.8%) may benefit from screening, compared to 2.3% of patients from the mutant cohort.

FGFR1 is a receptor tyrosine kinase that can be altered by mutation, chromosomal rearrangement or amplification in a diverse range of cancers, including lung and breast cancers. There is clinical data that supports the use of *FGFR*-targeted inhibitors, such as dovitinib and AZD4547, in patients with *FGFR1*-amplified breast and lung cancer. Similarly, patients with *FGFR1*-amplified CRC may also benefit from treatment (Chae *et al.,* 2017). There were (1.2% patients) six amplifications in *FGFR1*, all in the wild type cohort. Overall, there were significantly more mutations in *FGFR* genes (*FGFR1, FGFR2, FGFR3, FGFR4*) in the mutant cohort (102/571, 18%) compared to the wild type cohort (51/492, 10%) (p-value 0.001). *FGFR* mutations have been previously described in the context of breast, lung, and gastric cancers (Mathur *et al.,* 2014). In this study, we identified increased *FGFR* mutations in the mutant cohort and *FGFR1* amplifications in the wild type cohort, which has not been previously associated with the genomic landscape of CRC. Like *EGFR*, *FGFR* can also be used as a biomarker to predict response to therapy (Chae *et al.,* 2017). *FGFR1* amplifications and mutations were present in 3.25% of the wild type cohort and 3.7% of the mutant cohort, p-value 0.7.

EGFR regulates signaling pathways that control cellular proliferation. Overexpression and amplification of *EGFR* has been seen in the pathogenesis of several cancers, frequently NSCLC (Bethune *et al.,* 2010). *EGFR* amplification has also been observed in cases of CRC and can be used as a biomarker for treatment with anti-EGFR monoclonal antibodies (Yang *et al.,* 2012). This study identified 7 amplifications in *EGFR*, and they were all in the wild type cohort. This supports the use of copy number as

a biomarker for treatment selection in *KRAS/BRAF* wild type CRCs. *EGFR* mutations and amplifications were present in 5.3% of the both the mutant and wild type cohorts.

Structural Variant Analysis

Driver fusion events may also hold predictive value for therapy selection in CRC. In the past , targetable fusions in *ALK*, *RET*, and *ROS1* which are sensitive to kinase inhibitors, have only been observed in less than 1% of tumor samples (Aisner *et al.,* 2014; Rolle *et al.,* 2015). Structural variant analysis identified 1 patient from the wild type cohort with a *CAD-ALK* fusion. This patient qualifies for a current clinical trial at DFCI for treatment using entrectinib. *ALK* fusions are commonly seen in NSCLC, and rarely in CRC. As such, the clinical response to drugs such as Entrectinib or crizotinib in CRC cases has not been described. However, the positive response in lung cancers points at the possibility of promising therapeutic insights for CRCs as well. No other significant structural variants associated with current trials were identified in either mutant or wild type cohorts.

BRAF and *KRAS* Mutant Cases

There were 571(54%) total patients in the *KRAS/BRAF* mutant cohort. Of these, 32% (188 patients) had *BRAF* mutations and 66% (382 patients) had *KRAS* mutations. Both *BRAF* and *KRAS* are clinically actionable genes. *KRAS* mutations indicate a contraindication to the use of anti-*EGFR* agents, such as cetuximab and panitumumab. *BRAF* mutations can be targeted with in a combinatorial approach using selective RAF-inhibitors (dabrafenib and vemurafenib) together with an anti-EGFR antibody, with or without a MEK inhibitor. In addition, there are 3 clinical trials that target mutant *KRAS*

and *BRAF* using LY3009120, cetuximab, paclitaxel, irinotecan, alisertib, MLN2480, and MLN0128.

There were 16 cases (2.8% of mutant cases, 1.5% overall) in which patients had concomitant *KRAS* and *BRAF* mutations. This is interesting to note and has been reported in a case study in a highly aggressive subset of CRC which is in dire need of new therapeutic strategies. A single case study of a 29-year-old female with concomitant *KRAS* and *BRAF* mutations showed rapid disease progression leading to the patient's death within 3 months of diagnosis (Vitttal, Midditi, & Kumar 2017). Many clinics test for *KRAS* mutations and do not check *BRAF* if the patient is mutant for *KRAS*. *BRAF* is usually only screened in wild type *KRAS*. The poor prognosis of the case study and prevalence of other cases with concomitant mutations suggests that patients be screened for *BRAF*, even if they are mutant for *KRAS*. It may be possible to treat patients with *KRAS/BRAF* concomitant mutations with *BRAF* targeted therapy, such as vemurafenib. However, further studies will be needed to test the efficacy of this idea.

Tumor Mutational Burden

Cancer immunotherapy is still a relatively young and emerging field. Advances in precision medicine have contributed to the possibility of using one's own immune system to fight cancer. TMB can be used as a quantitative biomarker in immune-oncology to predict response to immunotherapy (Goodman *et al.,* 2017). Comprehensive genomic profiling tests can accurately measure TMB. Tumor cells high TMB may have more neoantigens, which are pieces of protein presented on the surface of the tumor cell as a result of acquired mutations that alter the expression of proteins. These neoantigens can be recognized by T-cells, inciting an anti-tumor immune response. Thus, high TMB may

indicate higher likelihood to respond to immune therapy. High TMB has been described as >20 mutations per Megabase in NSCLC (Goodman *et at.,* 2017). As such, >20 mutations per Megabase was used to describe high TMB in this study as well.

TMB was significantly higher in the mutant cohort as compared to the wild type cohort. There were 5.4% of patients with high TMB in the wild type cohort, compared to 16.6% from the mutant cohort, p-value $7.3x10^{-8}$. The median TMB in the mutant cohort was 9.45 mutations per Megabase (range 1.18 to 322.41) and 7.96 mutations per Megabase (range 1.18 to 148.76) in the wild type cohort. Higher TMB predicts a favorable response to PD-1/PD-L1 blockade in several cancer types (Goodman *et at.,* 2017). The results from this study indicate 16% patients from the mutant cohort and 5% patients from the wild type cohort may benefit from treatment with immunotherapy and should be screened for TMB.

Mutational Signatures

Mismatch repair (MMR) deficient tumors have hundreds to thousands of mutations. On average, there are 1700 mutations, compared to 70 in a typical cancer cell (Tontonoz, 2017). This results from the inability of cells to correct DNA base-pair mistakes, as the mismatch repair pathway is what facilitates this correction. In 2017, the FDA approved the use of pembrolizumab (Keytruda), an immune checkpoint inhibitor, for MMR deficient colorectal cancer. MMR status was deficient for 12.8% cases in the mutant cohort versus only 3.5% in the wild type cohort. There were significantly more MMR deficient cases in the mutant cohort (p-value 0.009), showing that *KRAS/BRAF* mutant CRC had a stronger mutational signature than wild type patients. However, regardless of which cohort the patient was in, all MMR deficient patients may be eligible

for treatment with this type of immunotherapy, even though it us unknown whether the presence of *KRAS* or *BRAF* mutation will make the patient respond better or less favorably.

Studies have shown that sporadic colorectal cancers with MMR-D display an enhanced immune surveillance mechanism and may be associated with a better prognosis than other CRCs (Scarpa *et al.,* 2015). As such, MMR deficiency was also assessed in the genes *MLH1, MSH2, MSH6, PMS2, BRCA1, BRCA2*, and *POLE*.

Approximately 5% of CRCs are hereditary in nature, such as HPNCC syndrome. Hereditary CRCs are characterized by mutations in MMR genes (*MLH1, MSH2, MSH6*, and *PMS2*) and microsatellite instability. There was no significant difference between the mutant and wild type cohort in frequency of these 4 genes (66 (13%) patients in the wild type cohort compared to 88 (15%) in the mutant cohort, p-value 0.4). Additionally, there was no significant difference in *BRCA1* and *BRCA2* between the two cohorts. *BRCA1* and *BRCA2* were altered in 95 (17%) patients in the mutant cohort and 73 (15%) patients in the wild type cohort, p-value 0.4.

Germline *POLE* mutations have also been associated with polyposis and colorectal predisposition. There were 21/571 (4%) patients with *POLE* mutations in the mutant cohort, and 9/492 (1.8%) in the wild type cohort, p-value 0.07. This result was also not significantly different.

Pathway Analysis

The RAS family of genes, including *KRAS*, *NRAS*, and *HRAS* produce proteins called GTPases. These proteins play a role in cell division, cell differentiation, and apoptosis. *BRAF*, *KRAS*, and *NRAS* are all part of the RAS/RAF/MEK/ERK pathway,

which is a signaling cascade to transmit signals from receptors to regulate gene expression and prevent apoptosis. Upstream receptors for this pathway include *EGFR* and *Flt*-3. Increased expression of this pathway is associated with poor prognosis. As expected, since the mutant cohort consisted of all *KRAS/BRAF* mutant cases, there were significantly more mutations in the RAS/RAF/MEK/ERK pathway (p value 0.00003). There were also significantly more mutations in the PIK3CA/AKT/MTOR pathway in the mutant cohort, driven by higher frequency of mutations in *AKT1*, *AKT2*, *AKT3*, *MTOR, PIK3CA, PTEN, TSC1*, and *TSC2* (p value 0.00005). This also confirms the mutational signature was stronger in the mutant cohort, as there was a higher frequency of MMR-D than compared to the wild type cohort.

Clinicopathologic Features

There were no statistically significant differences in clinicopathologic features between the mutant and wild type cohorts for significant results, *NRAS* mutations, and amplifications in *ERBB2*, *EGFR*, and *FGFR1*.

Candidates for Clinical Trials

After adjusting for *KRAS* and *BRAF* mutant and wild type targeted trials, this study identified 71% of the wild type cohort as potential candidates for currently available clinical trials at DFCI, based on targeted gene mutations, copy number alterations, and structural variations. Comparatively, there were 76% of patients in the mutant cohort that would qualify for trials as well. As expected, the frequency of patients that are eligible for clinical trials was similar in both mutant and wild type cohorts, if

KRAS and *BRAF* mutations were removed. This suggests that the clinical drivers appear to be equally dispersed between the two groups.

For many of the available trials, *KRAS/BRAF* status was not mentioned in the eligibility criteria. Because the status of these mutations is relevant to clinical response in some therapeutics, such as anti-EGFR antibodies, it may be significant in some trials as well. As knowledge of the driving events in colorectal cancer increases, the availability for potential therapeutics will also increase. Currently, there are therapeutics are have known efficacy in other cancer types, such as crizotinib in NSCLC and trastuzumab for breast cancer. The current clinical trials will uncover the effectiveness of several proposed therapeutics and positive response in colorectal cancer and may improve survival outcomes for many patients who otherwise had poor response to available treatment options.

Limitations and Future Research Directions

The mutant cohort was described as any patient harboring a mutation in *KRAS* or *BRAF*. There are a number of limitations to how the cohorts were divided. Firstly, any mutation in *BRAF* and *KRAS* specified assignment to the "mutant" cohort, regardless of whether the mutation was activating or not. Thus, another way to divide the cohorts would be to only assign activating *BRAF* and *KRAS* mutations to the mutant cohort. This would ensure that the cancers in the mutant cohort are driven by *KRAS*/*BRAF*. Secondly, *NRAS* mutations were not included in the mutant cohort, and were assigned to the wild

type cohort. *NRAS* is, however, still part of the RAS family of genes, and there is evidence to suggest that patients with *NRAS* mutated cancer will respond to targeted therapies in a similar way to *KRAS* mutated cancer. In addition, *NRAS* is mentioned in the NCCN treatment compendium for screening. Thus, *NRAS* cases (or just activating mutations thereof) could be included in the mutant cohort to account for this.

There were several patients in the overall cohort for which copy number analysis, structural variant analysis, tumor stage, laterality, mismatch repair status, and tumor mutational burden data was not available. The availability of this data may have some impact on the overall findings. Additionally, more comprehensive profiling may uncover additional recurrent alterations not identified by DNA sequencing, such as epigenetic changes and RNA mutations. As always, targeted sequencing has limitations in the number of genes interrogated (versus WES or WGS, for example)

Follow up studies to assess the efficacy and survival outcomes for patients that could potentially be enrolled in clinical trials will be a very important next step, especially for patients with actionable or targetable rare alterations- for example *ERBB2* amplifications and *FGFR1* amplifications

Concluding Remarks

KRAS/BRAF wild type CRC patients showed an increased frequency of *TP53* and *NRAS* mutations. In addition, wild type cases displayed a significantly higher occurrence of high amplifications in *ERBB2*, *FGFR1*, and *EGFR* and homozygous deletions in *PTEN* when compared to *KRAS/BRAF* mutant CRC cases. Furthermore, wild type cases had significantly lower tumor mutational burden, mismatch repair deficiency, and number of mutations in RAS/RAF/MEK/ERK and PIK3/AKT/MTOR pathways.

The genomic landscape of wild type CRC, as described in this study, confers the importance of genomic screening for all patients diagnosed with CRC, as all of the significant results hold clinical relevance. This study identified 71% of *KRAS/BRAF* wild type patients that would qualify for currently available clinical trials. Although no clinicopathologic features were associated with any results, this study emphasizes that profiling patient samples for alterations in a panel of potentially actionable gene mutations that can help clinicians select the appropriate therapeutic paradigm for patients. More comprehensive analyses using clinical outcomes data should aid in prioritizing clinical trials for colorectal cancer patients, as well as identifying new targets for therapy.

Appendix

List of Abbreviations

5-FU	5 Flourouracil
APC	Adenomatous Polyposis Coli
BRAF	v-RAF murine sarcoma viral oncogene homolog
CML	Chronic Myelogenous Leukemia
CRC	Colorectal Cancer
dMMR	Mismatch Repair Deficient
EGFR	Epidermal Growth Factor Receptor
FAP	Familial Adenomatous Polyposis
FFPE	Formalin-fixed, paraffin embedded
GIST	Gastrointestinal Stromal Tumors
HCC	Hepatocellular Carcinoma
HER-2	Human epidermal growth factor receptor 2 protein. Now ERBB2
HNPCC	Hereditary Nonpolyposis Colon Cancer
ICGC	International Cancer Genome Consortium
INDEL	Insertion/Deletion
IRB	International Review Board
IVD	In Vitro Diagnostic
KRAS	Kristen rat sarcoma 2 viral oncogene
MAPK	Mitogen Activated Protein Kinase
MMR	Mismatch Repair
MPS	Massively Parallel Sequencing
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability High
MSK-IMPACT	Integrated Mutation Profiling of Actionable Cancer Targets
MTOR	Mechanistic Target of Rapamycin
NCCN	National Comprehensive Cancer Network
NSAID	Non-Steroidal Anti-Inflammatory Drug
NSCLC	Non-small Cell Lung Cancer
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
TCGA	The Cancer Genome Atlas
TNM	Tumor, Node, Metastasis
WES	Whole Exome Sequencing

Stages of Cancer

Stage	Description
0	This is the earliest stage possible. Cancer hasn't moved from where it started; it's still restricted to the innermost lining of the colon. Stage 0 is also called Carcinoma in Situ.
I	Cancer has begun to spread, but is still in the inner lining. Stage I is also called Dukes A colon cancer.
II	Many of these cancers have grown through the wall of the colon and may extend into nearby tissue. They have not yet spread to the lymph nodes. Stage II is also called Dukes B colon cancer.
III	Cancer has spread to lymph nodes, but has not been carried to distant parts of the body. Stage III is also called Dukes C colon cancer.
IV	Cancer has been carried through the lymph system to distant parts of the body, most commonly lungs and liver. This is known as metastasis. Stage IV is also called Dukes D colon cancer.

Table 1. Description of the Stages of Cancer Progression

Typically, patients diagnosed in Stage 0 and Stage I have surgery as a treatment option. Stages II, III, and IV can be treated with chemotherapy, biologics, and radiation with or without surgery

FDA Approved Biological Therapies for Colorectal Cancer	
Biological Therapy	Gene Target
Cyramza (ramucirumab)	Anti-VEGFR2
Avastin **(**bevacizumab)	Anti-VEGF-A
Stivarga (regorafenib)	Anti-VEGF, Anti-EGFR
Erbitux (cetuximab)	Anti-EGFR
Vectibix (panitumumab)	Anti-EGFR
pembrolizumab (KEYTRUDA)	Anti-PD-1

Table 2. FDA-Approved Biological Therapies for Colorectal Cancer

Biological therapies work by targeting a specific gene or mutation. Currently, there are six biological therapies that have been approved by the FDA for specific use in colorectal cancer treatment. Biological therapies that have been approved for other cancer types (e.g. Crizotinib for NSCLC, trastuzumab for breast cancer) are under clinical investigation for response and efficacy in colorectal cancer.

Description of the Cohort

	***KRAS/BRAF* Mutant**	***KRAS/BRAF* Wild Type**
	N (%)	N (%)
Total number	571 (53.7%)	492 (46.3%)
Sex		
Male	274 (47.9%)	292 (59.3%)
Female	297 (52%)	200 (42.4%)
Age		
Median	56.06	57.88

Table 3. Description of the Cohort

The overall cohort contained 1063 colorectal cancer patients, with 53.7% patients harboring mutations in KRAS or BRAF oncogenes. 46.3% patients were wild type for these genes. There was no significant difference in the proportion of males and females in both groups and the median age.

Detailed Cancer Types

Cancer Type	Frequency	
	Mutant	**WT**
Colon Adenocarcinoma	98.96%	66.00%
Rectal Adenocarcinoma		20.93%
Colorectal Adenocarcinoma		11.27%
Mucinous Adenocarcinoma of the Colon and Rectum		1.21%
Esophageal Adenocarcinoma		0.20%
Signet Ring Cell Adenocarcinoma of the Colon and Rectum		0.20%
Melanoma		0.20%
Breast Invasive Ductal Carcinoma	0.35%	
Oropharynx Squamous Cell Carcinoma	0.17%	
Neuroendocrine Carcinoma, NOS	0.17%	
Adenocarcinoma, NOS	0.17%	
Small Cell Lung Cancer	0.17%	

Table 4. Detailed Cancer Types

The majority of cases (>98%) presented with colon/rectal or colorectal adenocarcinoma as the primary diagnosis. A few cases presented with other cancer types due to metastasis. The primary cancer diagnosis for these patients was CRC, however these patients had metastatic recurrences in other sites, such as breast and esophagus.

Top 25 Commonly Mutated Genes in Mutant and Wild Type Cohort				
Gene	**# Cases in Mutant**	**Frequency**	**# Cases in Wild Type**	**Frequency**
APC	424	73.23%	397	79.88%
TP53	383	67.01%	379	76.26%
KMT2D	352	60.79%	66	13.28%
PIK3CA	156	26.94%	77	15.49%
SOX9	188	32.92%	53	10.66%
ARID1A	110	19.00%	52	10.46%
FBXW7	102	17.62%	50	10.06%
SMAD4	100	17.27%	53	10.66%
ATM	95	16.41%	46	9.26%
BRCA2	88	15.20%	52	10.46%
TCF7L2	86	14.85%	54	10.87%
ARID1B	79	13.64%	49	9.86%
NOTCH1	78	13.47%	42	8.45%
NRAS	66	11.40%	48	9.66%
PRKDC	66	11.40%	42	8.45%
RECQL4	64	11.05%	38	7.65%
GLI2	64	11.05%	35	7.04%
GLI3	62	10.71%	34	6.84%
GNAS	60	10.36%	35	7.04%
CREBBP	59	10.19%	30	6.04%
EP300	59	10.19%	29	5.84%
TSC2	59	10.19%	30	6.04%
ERBB2	58	10.02%	29	5.84%
ERBB4	53	9.15%	28	5.63%
SETD2	53	9.15%	28	5.63%

Table 5. Top 25 Gene Mutations, Mutant and Wild Type

Difference in Mutational Frequency Mutated Genes			
Gene Name	**# Mutant**	**# Wild Type**	**P-Value**
ALK	53	24	0.007
APC	424	397	0.23
ARID1A	86	52	0.04
ARID1B	79	49	0.06
ATM	110	46	2.58E-05
BRCA2	64	52	0.75
CREBBP	59	30	0.01
EP300	50	29	0.08
FBXW7	88	50	0.01
GLI2	51	35	0.29
GLI3	35	34	0.61
GNAS	49	35	0.39
KMT2D	102	66	0.06
NOTCH1	66	42	0.12
NRAS	13	48	3.85E-07
PIK3CA	156	77	5.07E-05
PRKDC	78	42	0.01
RECQL4	42	38	0.82
SETD2	46	28	0.14
SMAD4	95	53	0.01
SOX9	100	53	0.003
TCF7L2	66	54	0.77
TP53	352	379	0.002
TSC2	38	30	0.72

P<0.05
Significantly
Different

Table 6. Difference in Mutational Frequencies of Mutated Genes from both Cohorts

Gene	# Mutant Cases	%	# Wild Type Cases	%	P Value	Clinical Relevance
AKT1	25	4.32%	4	0.80%	0.0004	AKT-targeted inhibitors such as AZD-5363
AKT2	13	2.25%	2	0.40%	0.01	AKT-targeted inhibitors such as AZD-5363
AKT3	12	2.07%	7	1.41%	0.4	AKT-targeted inhibitors such as AZD-5363
ALK	53	9.15%	24	4.83%	0.007	*ALK* rearrangements - *ALK* kinase inhibitors, such as crizotinib and ceritinib
ATM	110	19.00%	46	9.26%	2.58E-05	ATM kinase inhibitor
BRAF	188	25.91%	0	0.00%	0	Selective RAF-inhibitors dabrafenib and vemurafenib together with an anti-EGFR antibody with or without a MEK-inhibitor, such as the doublet vemurafenib plus panitumumab or the triplet dabrafenib, trametinib, plus panitumumab
BRCA1	40	6.91%	23	4.63%	0.11	PARP inhibitors rucaparib, olaparib and niraparib
EGFR	30	5.18%	19	3.82%	0.29	EGFR tyrosine kinase inhibitors (TKIs) erlotinib, afatinib and gefitinib
ERBB2	38	6.56%	29	5.84%	0.62	Mutations - neratinib; amplifications - HER2 inhibitors
FLT3	30	5.18%	15	3.02%	0.08	sorafenib
HRAS	6	1.04%	1	0.20%	0.07	Firm contraindication to the use of anti-EGFR agents such as cetuximab or panitumumab
IDH1	12	2.07%	8	1.61%	0.57	IDH1-targeted inhibitors such as AG-120
KRAS	382	65.98%	0	0.00%	0	Firm contraindication to the use of anti-EGFR agents such as cetuximab or panitumumab
MAP2K1	9	1.55%	15	3.02%	0.11	MEK-inhibitors such as cobimetinib, trametinib and selumetinib
MTOR	53	9.15%	21	4.23%	0.002	everolimus
NRAS	13	2.25%	48	9.66%	3.85E-07	Firm contraindication to the use of anti-EGFR agents such as cetuximab or panitumumab
TSC1	28	4.84%	13	2.62%	0.06	everolimus
TSC2	38	6.56%	30	6.04%	0.72	everolimus

Table 7. Frequency and Comparison of Clinically Actionable Genes

Protocol #	Gene Mutations	Wild Type Genes	CNV Genes	SV Genes	Drugs
06-068	*MET*		*ALK, MET*	*ROS1, MET, NTRK1, ALK*	PF-02341066, ITRACONAZOLE, RIFAMPIN, MIDAZOLAM, KETOCONAZOLE
08-007	*MET*		*AXL, MET*	*TFE3, MET, FOXO1, EWS, AXL, MITF*	MGCD265
11-010		*PIK3CA, KRAS*	*PIK3CA*		GDC-0032
11-490			*PIK3CA*		BYL719, ARRY-438162
12-085			*MYCN*		GSK525762
12-429	*BRAF*				GSK2118436
13-010					BVD 523
13-180			*ALK*	*ALK*	LDK378
13-194		*TP53*			CGM097
13-251			*FGFR1, FGFR3, FGFR2*	*FGFR2, FGFR1, FGFR3*	DEBIO 1347
13-300			*PTEN, PIK3CB*		ABIRATERONE ACETATE, AZD2014, AZD8186
13-505	*NRAS, KRAS*				NAVITOCLAX, TRAMETINIB
13-523	*BRAF*				LY3009120
13-615	*ERBB2*		*EGFR*		NERATINIB, FULVESTRANT, PACLITAXEL
14-118		*TP53*	*MDM2*		AMG 232
14-158	*NRAS, KRAS*	*NRAS, KRAS*			BMS-663513, CETUXIMAB
14-186	*BRAF*	*NRAS, KRAS*			DABRAFENIB, TRAMETINIB, AT13387
14-301	*KRAS*	*KRAS*			MPDL3280A, COBIMETINIB
14-455	*EGFR, KRAS*		*MYC*		CABOZANTINIB, PACLITAXEL, CB-839, EVEROLIMUS, ERLOTINIB
14-464			*FGFR4*		FGF401, PDR001
14-487	*AKT1*				BAY1125976
14-524		*KRAS*			MGD007
14-537	*BRAF*				CETUXIMAB, PACLITAXEL, IRINOTECAN, ALISERTIB, MLN2480, MLN0128

14-574		*NRAS, KRAS*			BEVACIZUMAB, IRINOTECAN, PLACEBO, CETUXIMAB
14-580	*IDH1*				IDH305
15-079	*BRAF*			*BRAF*	DABRAFENIB, TRAMETINIB
15-084		*KRAS*		*NTRK1, NTRK3, NTRK2, ROS1*	DS-6051B
15-129				*RSPO*	LGK974
15-281			*CCNE1, MCL1*		CYC065
15-289		*TP53*			HDM201
15-435				*ROS1, NTRK1, NTRK3, NTRK2, ALK*	ENTRECTINIB
15-454				*NTRK3, NTRK2, NTRK1*	LOXO-101
15-495			*KIT, PDGFRA*	*PDGFRA, KIT*	DCC-2618
15-524			*NF1*	*FLT3*	COBIMETINIB
16-045				*ROS1, NTRK1, NTRK3, NTRK2, ALK*	ENTRECTINIB
16-076	*FGFR4*		*FGF19, FGFR4*		BLU-554
16-094				*NTRK3, NTRK2, NTRK1*	LOXO-101
16-254			*CCND1, CDKN2A, CCND2, CCNE1, CCND3, CDK6, CDK4*		PALBOCICLIB
16-281			*MYC, CCNE1, RB1*		LY2606368
16-374				*MET, NTRK3, NTRK2, NTRK1*	MERESTINIB
16-494			*JAG1, NOTCH2, NOTCH3, NOTCH1, JAG2*		LY3023414, LY3039478, GEMCITABINE, TALADEGIB, CARBOPLATIN, ABEMACICLIB, CISPLATIN
16-499	*PIK3CA*		*PIK3CA, PTEN*		PF-05212384, PALBOCICLIB
16-534			*CHEK2, RAD51D, BRCA2, BRCA1, PALB2, ATM*		BTP-114
16-573			*BRCA1, BRCA2*		OLAPARIB, PREXASERTIB
16-597			*JAG1, NOTCH2, NOTCH3, NOTCH1, JAG2*		LY3039478, PREDNISONE
16-711			*RAF1, ERBB2*		ONALESPIB, AT7519M

16-750	*ERBB2, GNAQ, AKT1, DDR2, PIK3CA, NRAS, SMO, PTEN, BRAF, GNA11, EGFR, PTCH1, FGFR2, FGFR3, FGFR1*		*PTEN, BRCA2, MLH1, RB1, CCND3, FGFR3, CCND1, TSC1, BRCA1, CDK4, FGFR2, NF1, NF2, MSH2, MET, CDK6, PIK3CA, MTOR, FGFR1, TSC2, ERBB2, CCND2*	*MET, NTRK3, ROS1, FGFR2, NTRK2, BRAF, TSC2, TSC1, FGFR1, MTOR, FGFR3, NTRK1, ALK*	AZD5363, DASATINIB, PERTUZUMAB, PALBOCICLIB, AZD1775, DABRAFENIB, LOXO-101, VS-6063, TRAMETINIB, AFATINIB, AZD9291, SUNITINIB, GDC-0449, CRIZOTINIB, NIVOLUMAB, TRASTUZUMAB, BINIMETINIB, AZD4547, TASELISIB, FULVESTRANT, MLN0128
17-016			*ATM*		VELIPARIB, CISPLATIN, VX-970
17-124	*BRAF*	*KRAS*			BINIMETINIB, IRINOTECAN, FOLFIRI, CETUXIMAB, ENCORAFENIB
17-236			*MYC*		SY-1365
17-241	*RET*			*RET*	LOXO-292
17-716			*RET, MET, AXL*	*RET, MET, AXL*	CABOZANTINIB

Table 8. Current Clinical Trials at DFCI, Gene Targets, and Corresponding Drugs

Copy Number Analysis (798 total CRC patients)			
	High Amplifications		
Gene	Mutant	Wild Type	P Value
CCND1	2 (33%)	4 (66%)	0.29
CCND2	5 (50%)	5 (50%)	0.77
CCND3	2 (100%)	0	0.19
CDK6	0	1 (100%)	0.27
EGFR	0	7 (100%)	0.003
ERBB2	2 (14%)	12(86%)	0.002
FGFR1	0	6 (100%)	0.007
MYC	17 (59%)	12 (41%)	0.66
	Low Amplifications		
ERBB2	91 (69%)	41(31%)	0.0009
	Single Deletions		
MET	4 (44%)	5 (55%)	0.53
PTEN	62 (78%)	17 (22%)	0.00002
	Double Deletions		
CDKN2A	4 (40%)	6 (60%)	0.35
SMAD2	4 (57%)	3 (43%)	0.89
SMAD4	12 (75%)	4 (25%)	0.1
TP53	2 (66%)	1 (33%)	0.67

Table 9. Copy Number Analysis, 798 Colorectal Cancer Patients

Significant differences in frequency between the mutant and wild type cohort were identified in high amplifications in ERBB2, EGFR, and FGFR1. There was no significant difference in the number of HA in *CCNDI*, *CCND2*, *CCND3*, *CCNE1* and *MYC* between the two cohorts.

Genes and Associated Pathways		
RAS/RAF/MEK/ERK	**PI3K/AKT/MTOR**	**RTKs**
BRAF	*AKT1*	*ALK*
HRAS	*AKT2*	*ARAF*
KRAS	*AKT3*	*AXL*
MAP2K1	*MTOR*	*EGFR*
MAP2K4	*PIK3C2B*	*EPHA3*
MAP3K1	*PIK3CA*	*EPHA5*
MAPK1	*PIK3R1*	*EPHA7*
MITF	*PTEN*	*ERBB2*
NF1	*TSC1*	*ERBB3*
NF2	*TSC2*	*ERBB4*
NRAS		*FGFR1*
RAF1		*FGFR2*
		FGFR3
		FGFR4
		FLT1
		FLT3
		FLT4
		IGF1R
		KIT
		MET
		PDGFRA
		PDGFRB
		PTPN11
		RET
		ROS1
		SH2B3
		ABL1

Table 10. Genes and Associated Pathways

3 actionable molecular pathways (RAS/RAF/MEK/ERK, PIK3CA/AKT/MTOR, and RTK (receptor tyrosine kinase) and the associated genes were assessed. Comparing the two cohorts, there were significantly more mutations in the RAS/RAF/MEK/ERK pathway (p value $3x10^{-4}$) and the PIK3CA/AKT/MTOR pathway (p value $5x10^{-5}$) in the mutant cohort compared to the wild type cohort. There was no significant difference in the number of mutations in the RTK pathway between both cohorts.

Clinicopathologic Features for 14 Patients with *ERBB2* High Amplifications								
Cohort	Laterality	Stage	Age	Gender	Cancer Diagnosis	Cancer Type	Tumor Purity	Recurrent Hotspot Mutations
Mutant	Not a paired site	null	41	M	Metastatic Recurrence - liver	Colon Adenocarcinoma	90	*KRAS, TP53*
Mutant			82	M	Primary	Rectal Adenocarcinoma	40	*KRAS, XPO1*
Wild Type			33	F	Primary	Colon Adenocarcinoma	20	*TP53*
Wild Type	Not a paired site	3	47	F	Primary	Colorectal Adenocarcinoma	40	*TP53*
Wild Type	Not a paired site	4	51	F	Primary	Colon Adenocarcinoma	50	None
Wild Type	Not a paired site	null	55	F	Primary	Colorectal Adenocarcinoma	50	*TP53*
Wild Type	Not a paired site	4	56	F	Metastatic Recurrence - lung	Rectal Adenocarcinoma	20	*TP53*
Wild Type			56	F	Primary	Colon Adenocarcinoma	50	None
Wild Type	Not a paired site	4	81	F	Primary	Colon Adenocarcinoma	30	*TP53*
Wild Type	Not a paired site	3	27	M	Primary	Rectal Adenocarcinoma	80	*TP53*
Wild Type			48	M	Primary	Rectal Adenocarcinoma	40	None
Wild Type	Not a paired site	4	49	M	Primary	Rectal Adenocarcinoma	50	*TP53*
Wild Type			72	M	Primary	Colon Adenocarcinoma	40	None
Wild Type			78	M	Metastatic Recurrence - lung	Colon Adenocarcinoma	40	None

Table 11. Clinicopathologic Features for 14 Patients with *ERBB2* Amps

Clinicopathologic Features for 7 Patients with *EGRF* High Amplifications						
Cohort	Age	Gender	Cancer Diagnosis	Cancer Type	Tumor Purity	Recurrent Hotspot Mutations
Wild Type	60	Male	Primary	Colon Adenocarcinoma	70	*BRCA2, APC, TP53*
Wild Type	85	Male	Primary	Colon Adenocarcinoma	50	*APC, TP53*
Wild Type	46	Male	Local Recurrence	Colon Adenocarcinoma	60	*APC, SMAD4,*
Wild Type	35	Male	Metastatic Recurrence	Rectal Adenocarcinoma	20	*TP53, ERBB2*
Wild Type	67	Female	Primary	Colorectal Adenocarcinoma	60	*APC, TP53*
Wild Type	39	Female	Metastatic Recurrence	Colon Adenocarcinoma	60	*APC, TP53*
Wild Type	31	Female	Metastatic Recurrence	Colorectal Adenocarcinoma	100	*TP53, PIK3CA*

Table 12. Clinicopathologic Features for 7 Patients with *EGFR* High Amps

Clinicopathologic Features for 6 Patients with *FGFR1* High Amplifications

Cohort	Age	Gender	Cancer Diagnosis	Cancer Type	Tumor Purity	Recurrent Hotspot Mutations
Wild Type	54	Male	Primary	Rectal Adenocarcinoma	20	*APC*
Wild Type	58	Male	Primary	Colon Adenocarcinoma	65	*TP53, PIK3R1, APC*
Wild Type	46	Male	Metastatic Recurrence	Colon Adenocarcinoma	30	*XPO1, APC*
Wild Type	48	Male	Metastatic Recurrence	Colorectal Adenocarcinoma	70	*APC, TP53*
Wild Type	47	Male	Primary	Colon Adenocarcinoma	40	*APC, TP53*
Wild Type	39	Female	Primary	Colon Adenocarcinoma	30	*APC*

Table 13. Clinicopathologic Features for 6 Patients with *FGFR1* High Amps

35 Mutated Genes, 12 Cases with *ERBB2* High Amps.		
Gene	**# Patients with Mutations**	**Frequency of Mutations**
TP53	12	91.67%
APC	12	83.33%
REL	4	33.33%
STAG1	3	25%
PDGFRA	2	16.67%
STAG2	2	16.67%
PTCH1	2	16.67%
CBLB	2	16.67%
EPHA5	2	16.67%
ERBB3	1	8.33%
ABL1	1	8.33%
PDGFRB	1	8.33%
ZRSR2	1	8.33%
ETV6	1	8.33%
MCL1	1	8.33%
IKZF1	1	8.33%
EXT2	1	8.33%
MEN1	1	8.33%
FANCA	1	8.33%
FANCE	1	8.33%
FANCF	1	8.33%
FANCG	1	8.33%
ARID1B	1	8.33%
RECQL4	1	8.33%
FGFR3	1	8.33%
SLITRK6	1	8.33%
KDM6A	1	8.33%
XPO1	1	8.33%
SETD2	1	8.33%
IGF1R	1	8.33%
SETBP1	1	8.33%
FLCN	1	8.33%
PRKDC	1	8.33%
ATM	1	8.33%
SOX9	1	8.33%

Table 14. Top 35 Mutated Genes, 12 Patients with *ERBB2* High Amps.

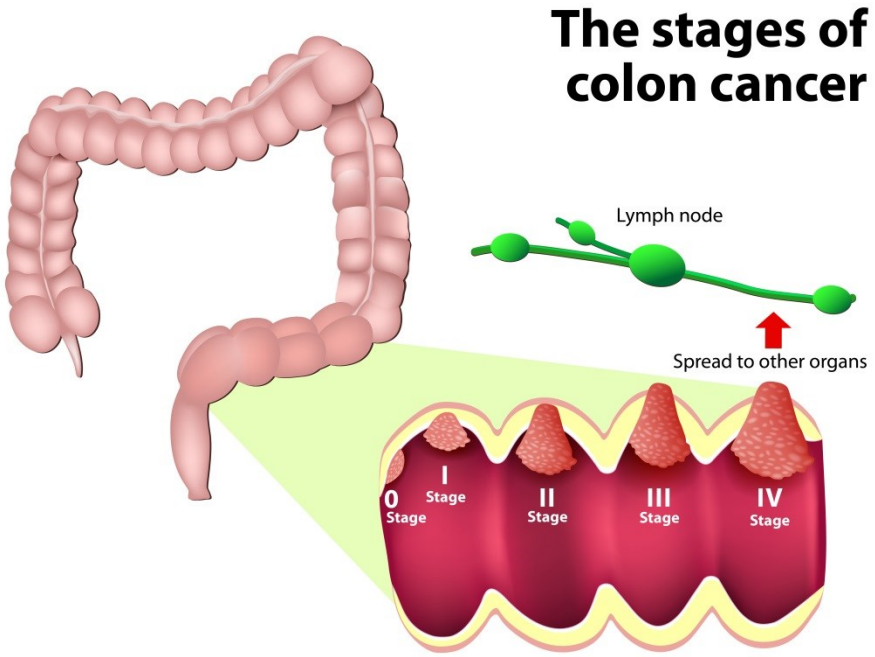


Figure 1. Stages of Colon Cancer

First, cancerous cells begin to form on the inner lining of the large intestine. Next, the cancer cells imbed and grow in the wall of the colon or lymph vessels. From there, the cancer cells can penetrate the blood or the lymph vessels. Finally, cancer cells spread into the nearby lymph nodes and can be transferred to other sites in the body (http://www.genome.gov, 2016).

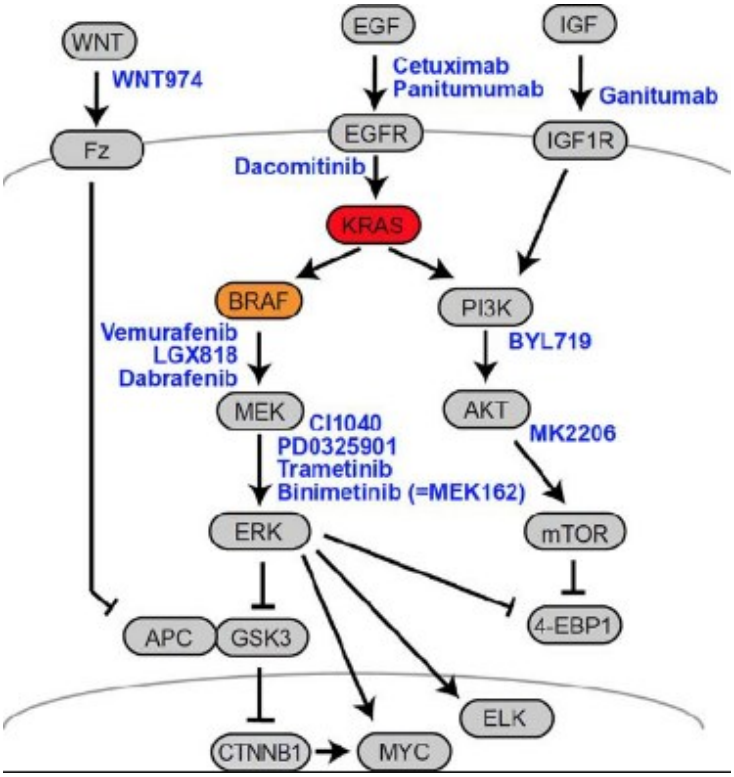


Figure 2. MAPK Pathway and Feedback Mechanisms

A schematic representation of the MAPK, PI3K and Wnt-APC-β-Catenin signaling axes is given, along with signaling connections. Major positive interactions are given as black arrows, while inhibitory interactions are given as red blocked lines. Solid lines indicate molecular interactions, whereas dotted lines indicate transcriptional control. Names frequently refer to a representative member of a multiprotein family.

Oncotarget. 2015 Aug 28; 6(25): 20785–20800.
(Morkel *et al.,* 2015)

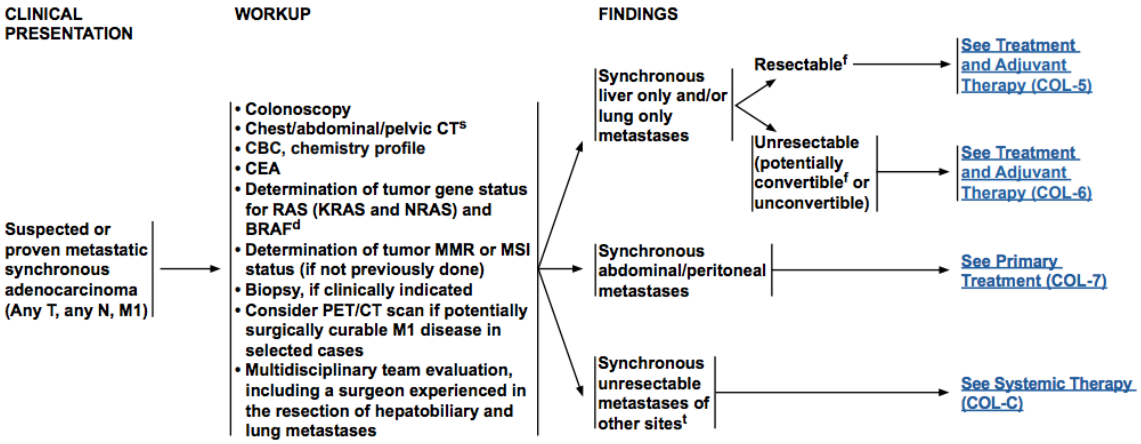


Figure 3. NCCN CRC Treatment Schema

NCCN Version 2.2107. National Comprehensive Cancer Network
http://www.nccn.org

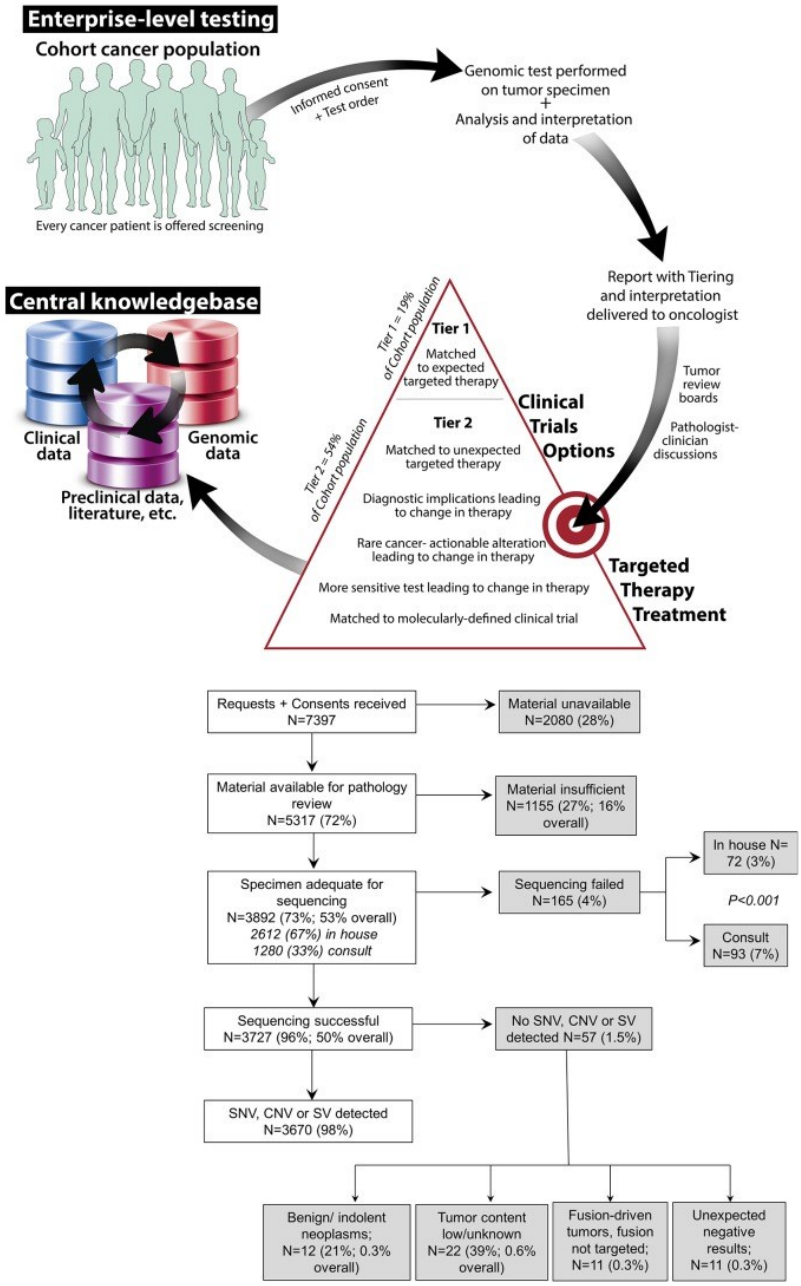


Figure 4. PROFILE, Programmatic Approach

For consented patients, a cancer specimen is genomically profiled in a CLIA laboratory. Results are tiered by a team that interprets pathology, incorporating information from each patient's electronic health record and provided to the patient's treating physician(s). Genomic, pathologic, and clinical data are deposited in a central knowledge base that can link to full clinical annotation. The knowledge base can be queried to facilitate development and enrollment of basket trials and inform tumor board discussions.
JCI Insight. 2016 Nov 17; 1(19): e87062.
(Sholl *et al.,* 2016)

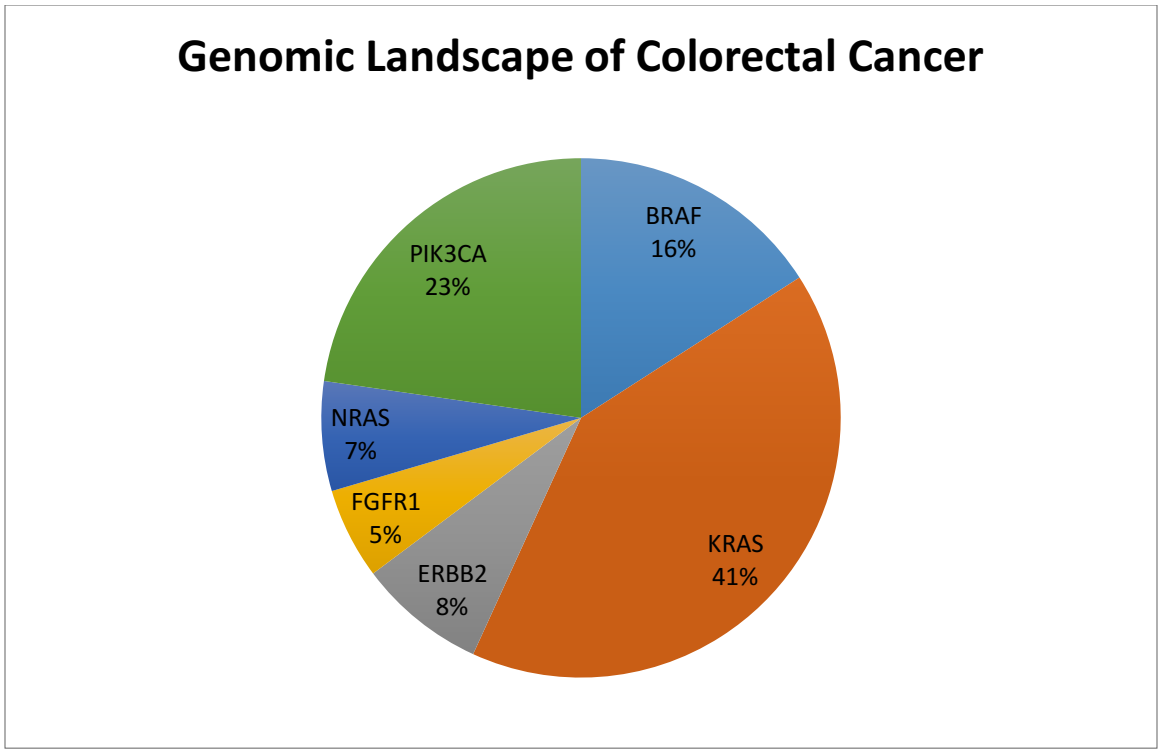


Figure 5. Genomic Landscape of Colorectal Cancer, Overall CRC cohort.

A total of 571 (53.7%) cases fell into the *KRAS/BRAF* mutant category. Together, mutations in *KRAS* and *BRAF* account for about half of all CRC cases. Other drivers drive the remaining half. Now, *ERBB2*, *FGFR1*, *NRAS*, and *PIK3CA* have also been described.

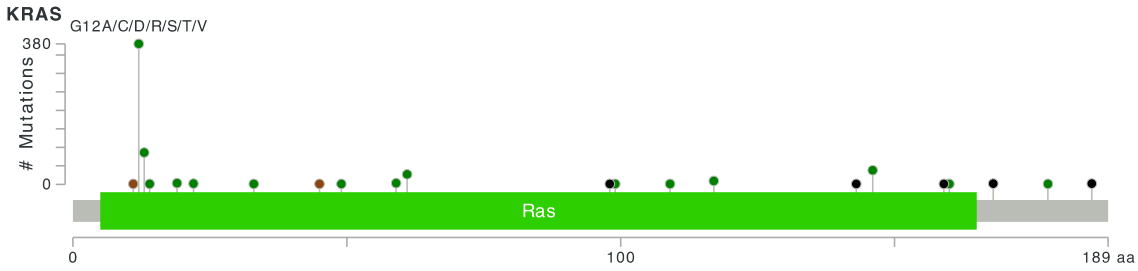


Figure 6. Spectrum of Mutations, *KRAS*

95% of KRAS mutations were known activating mutations, such as G12A and G13D

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

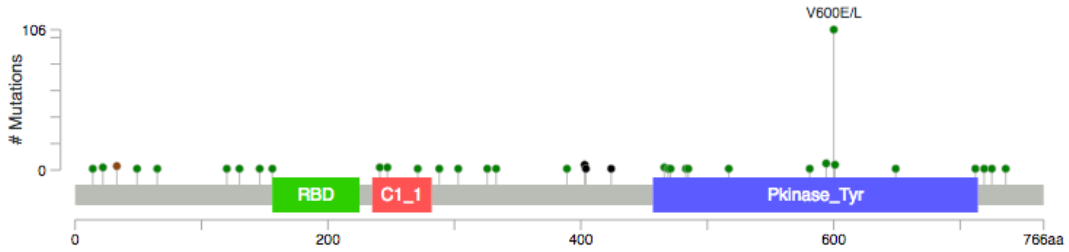
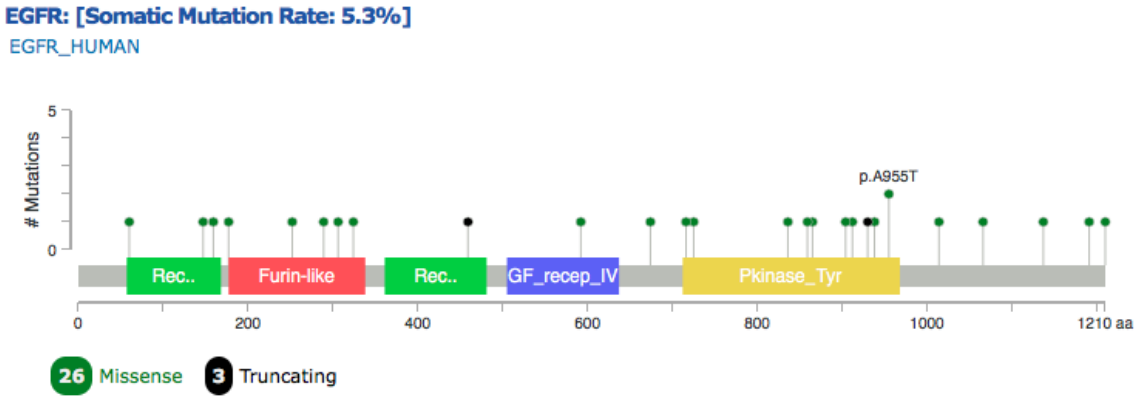


Figure 7: Spectrum of Mutations, *BRAF*

63% of total BRAF mutations were the canonical activating V600E mutation

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

Mutant Cohort



Wild Type Cohort

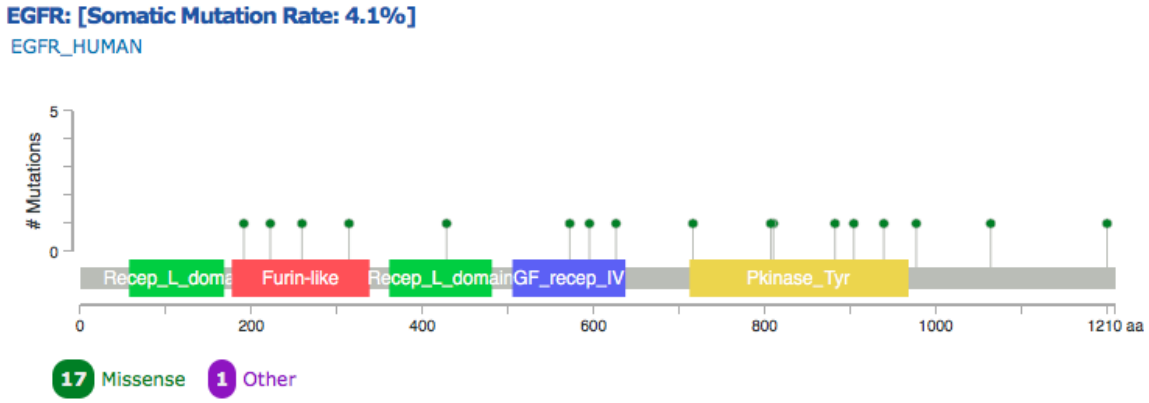
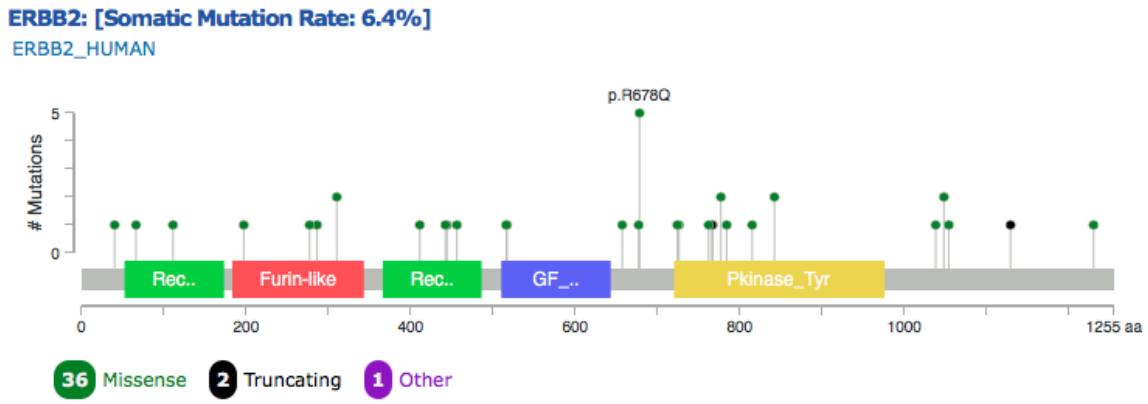


Figure 8: Spectrum of Mutations, *EGFR* (Mutant and Wild Type Cohorts

There was no significant difference in the frequency of *EGFR* mutations in both wild type and mutant cohorts (p value 0.29). There were 19/492 (3.8%) cases with *EGFR* mutations in the wild type cohort and 30/571(5.2%) cases with *EGFR* mutations in the mutant cohort. In addition, the overall spectrum of mutations for both cohorts was relatively similar, with no major differences noted

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

Mutant Cohort



Wild Type Cohort

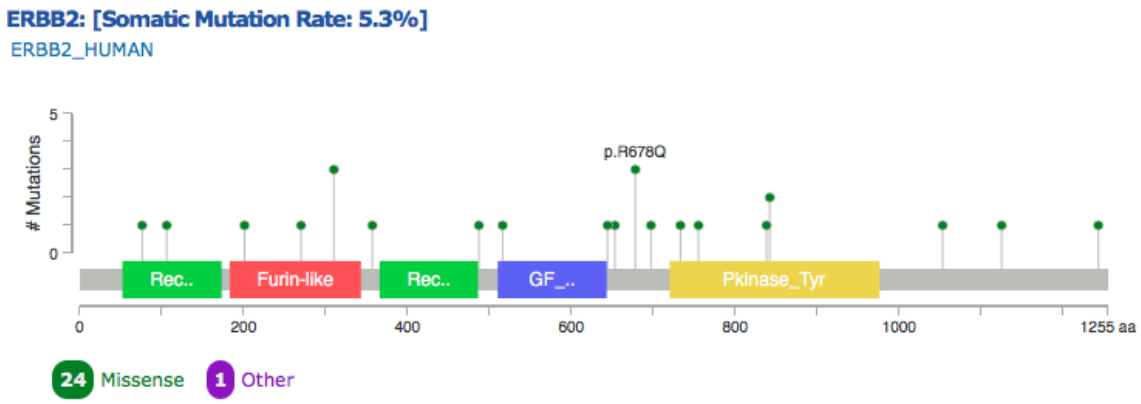
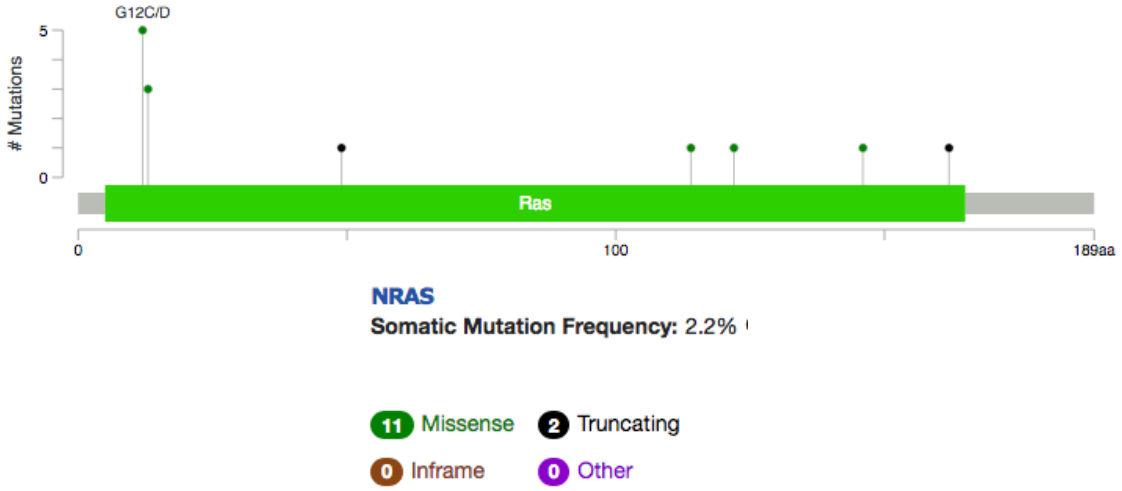


Figure 9: Spectrum of Mutations, *ERBB2* (Mutant and Wild Type Cohorts)

The difference in frequency of *ERBB2* (38/571 (6.6%) mutant vs. 29/492 (5.8%) wild type) between the two cohorts was not significant. Of the 29 cases with *ERBB2* mutations in the wild type cohort, 28 of the mutations were missense and 1 was a splice region mutation, which co-occurred with an *ERBB2* missense mutation in the same patient

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

Mutant Cohort



Wild Type Cohort

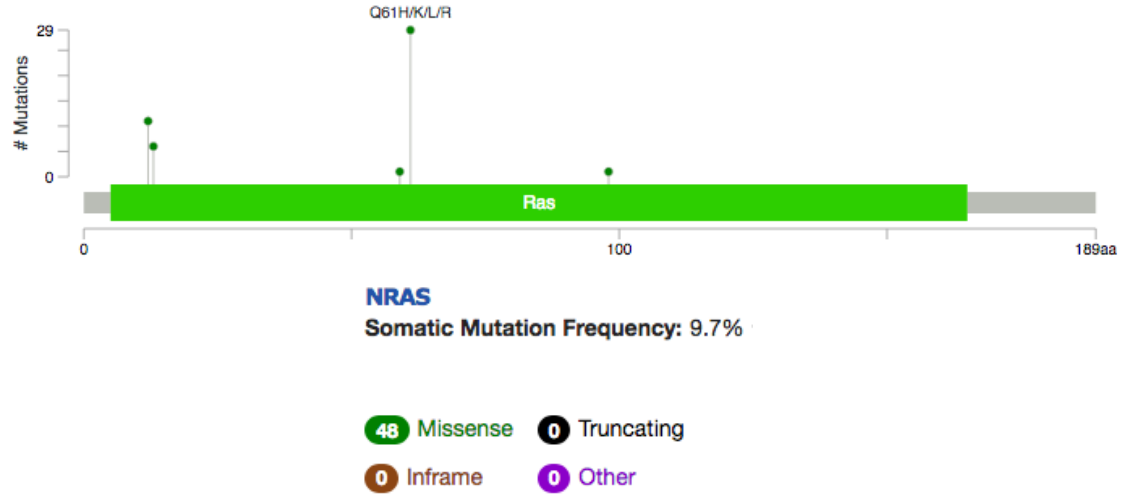
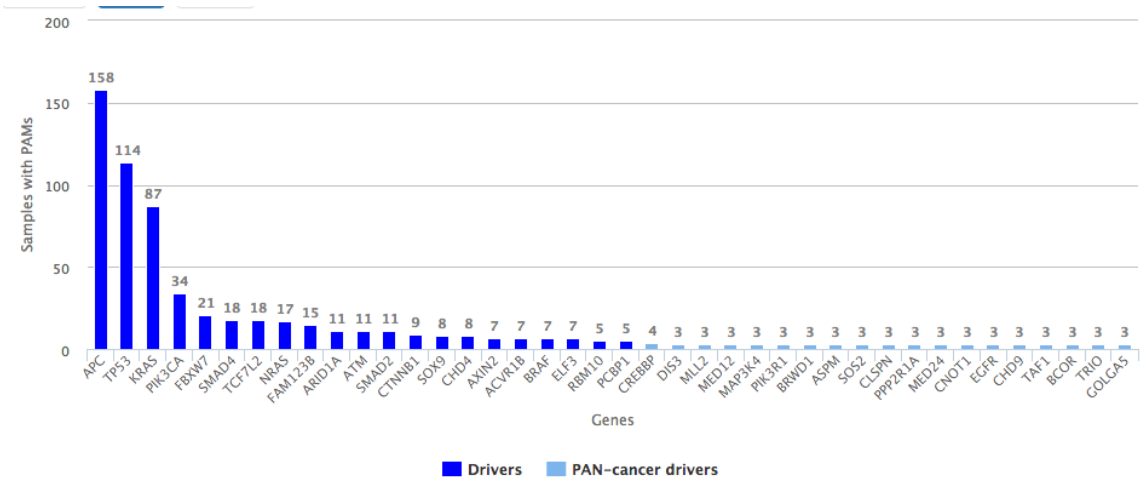


Figure 10: Spectrum of Mutations, *NRAS* (Mutant and Wild Type Cohorts)

Mutations in *NRAS* (13/571 (2%) mutant vs. 48/492 (9.7%) wild type) were more frequent in the wild type cohort (p value 3.86×10^{-07}). All of the *NRAS* mutations in the WT cohort were recurrent hotspot mutations, such as G12A, G12C, G13D, and A146T. These are all known oncogenic driving mutations

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

TCGA Top 50 Genes, CRC



This plot shows the most recurrently mutated **cancer driver genes in the Colorectal adenocarcinoma TCGA dataset**. Each bar of the histogram indicates the amount of samples with PAMs.

COSMIC, Top 20 Genes, CRC

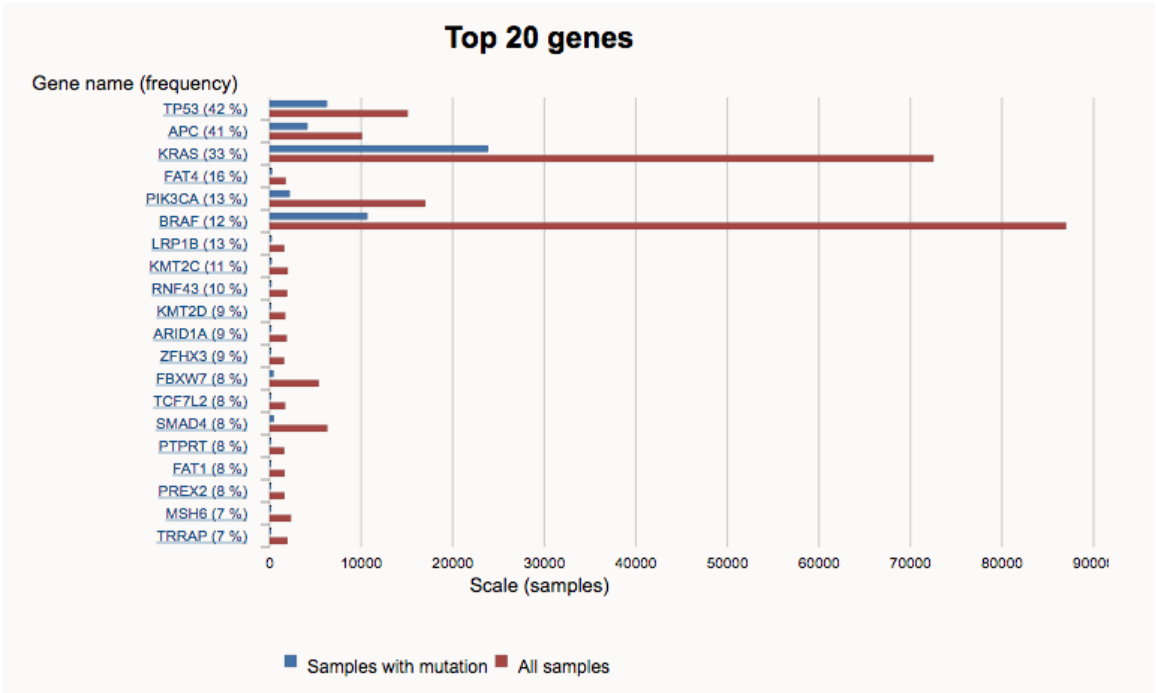
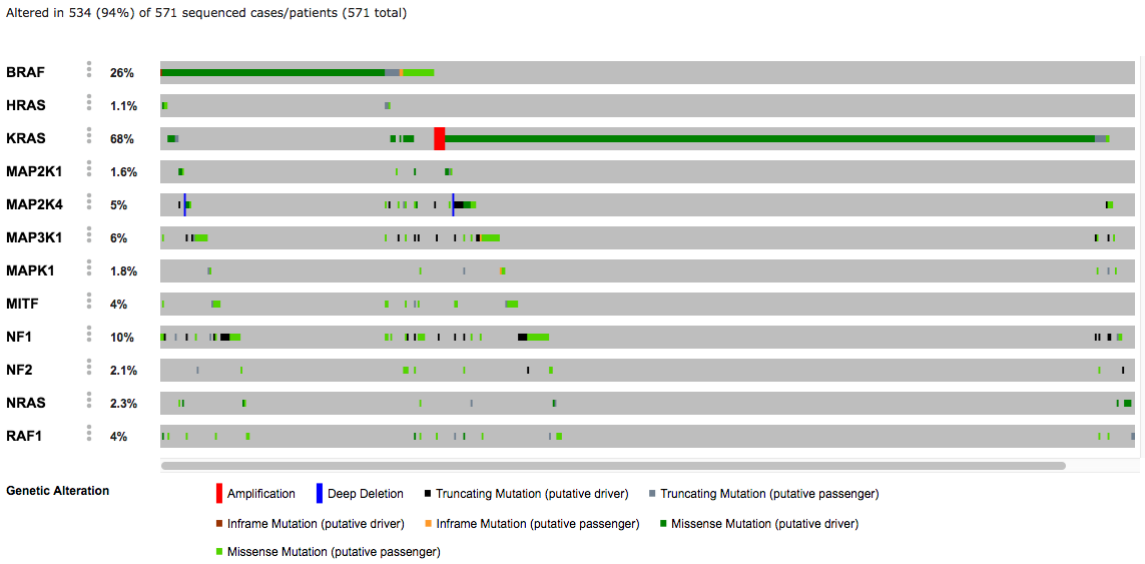


Figure 11. TCGA and COSMIC, Commonly Mutated Genes in Colorectal Cancer

The most commonly mutated genes in CRC reported by the TCGA and COSMIC include *APC*, *TP53*, *KRAS*, and *PIK3CA* among others. As expected, there were approximately 50% of cases wild type for *KRAS* and *BRAF* mutations, with 36% *KRAS* and 9% *BRAF* mutations present.
https://cancergenome.nih.gov/

http://cancer.sanger.ac.uk/cosmic

RAS/RAF/MEK/ERK Pathway Genes Altered in Mutant Cohort.



RAS/RAF/MEK/ERK Pathway Genes Altered in Wild Type Cohort.

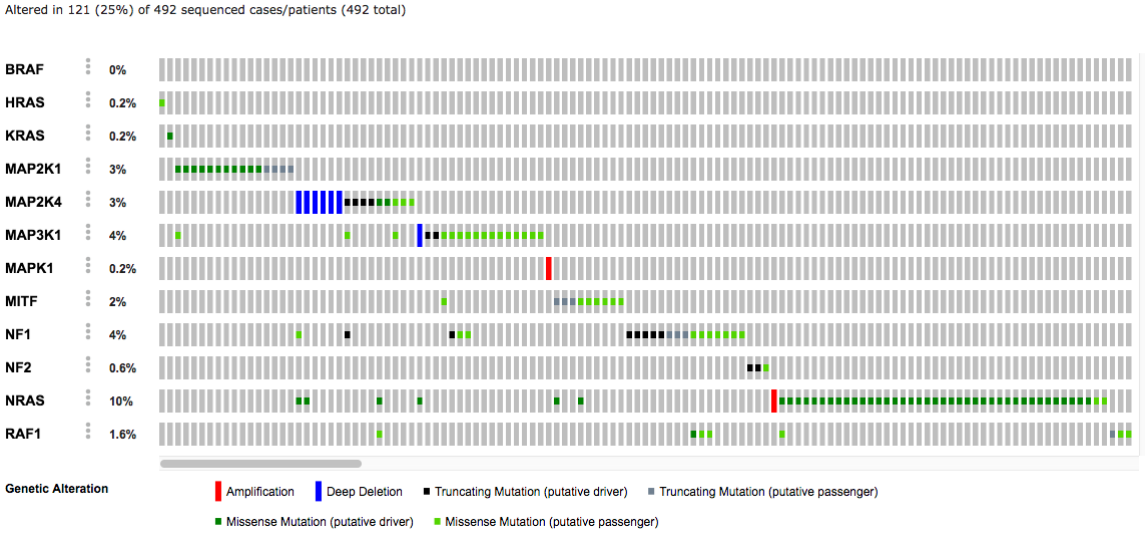
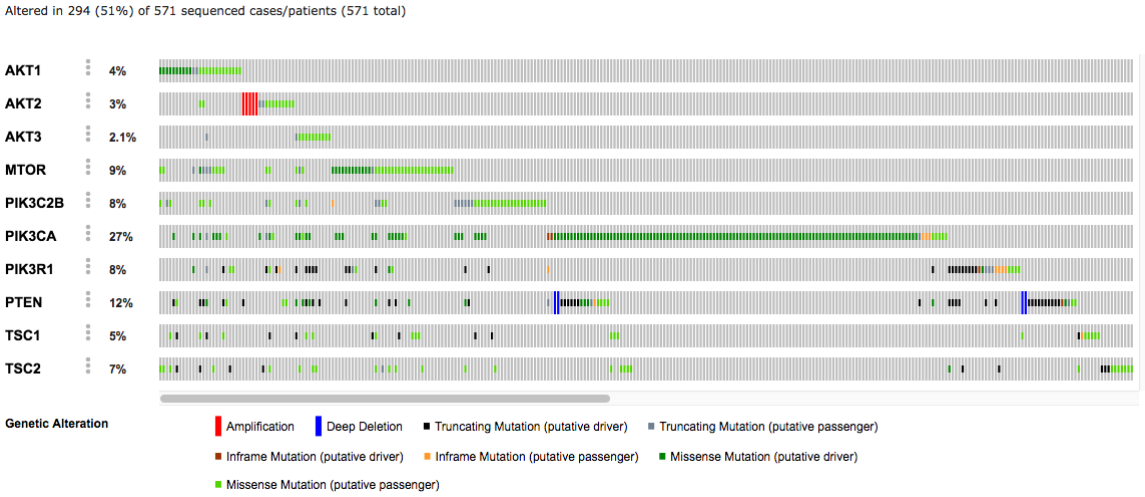


Figure 12. RAS/RAF/MEK/ERK Pathway Genes

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

PIK3CA/AKT/MTOR Pathway Genes Altered in Mutant Cohort



PIK3CA/AKT/MTOR Pathway Genes Altered in Wild Type Cohort

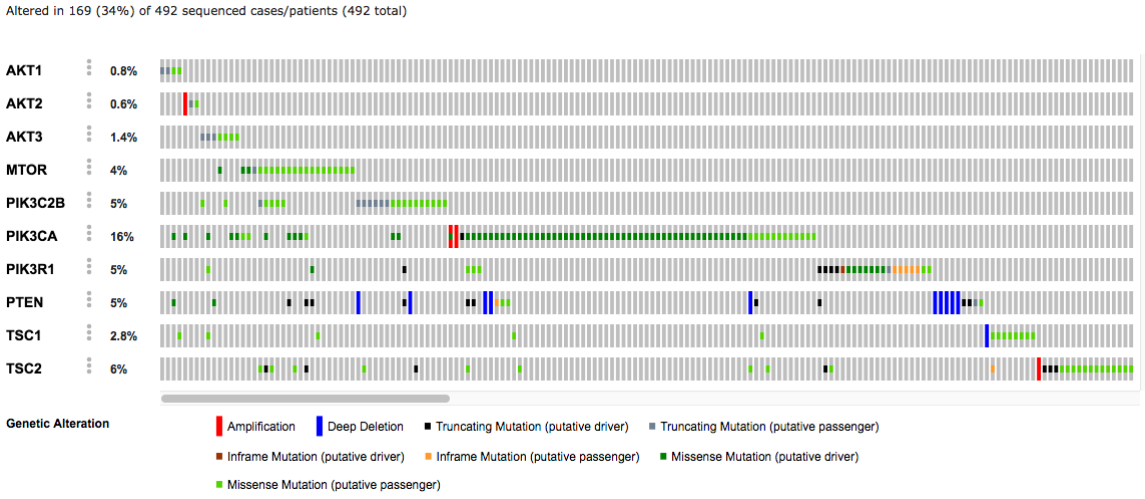
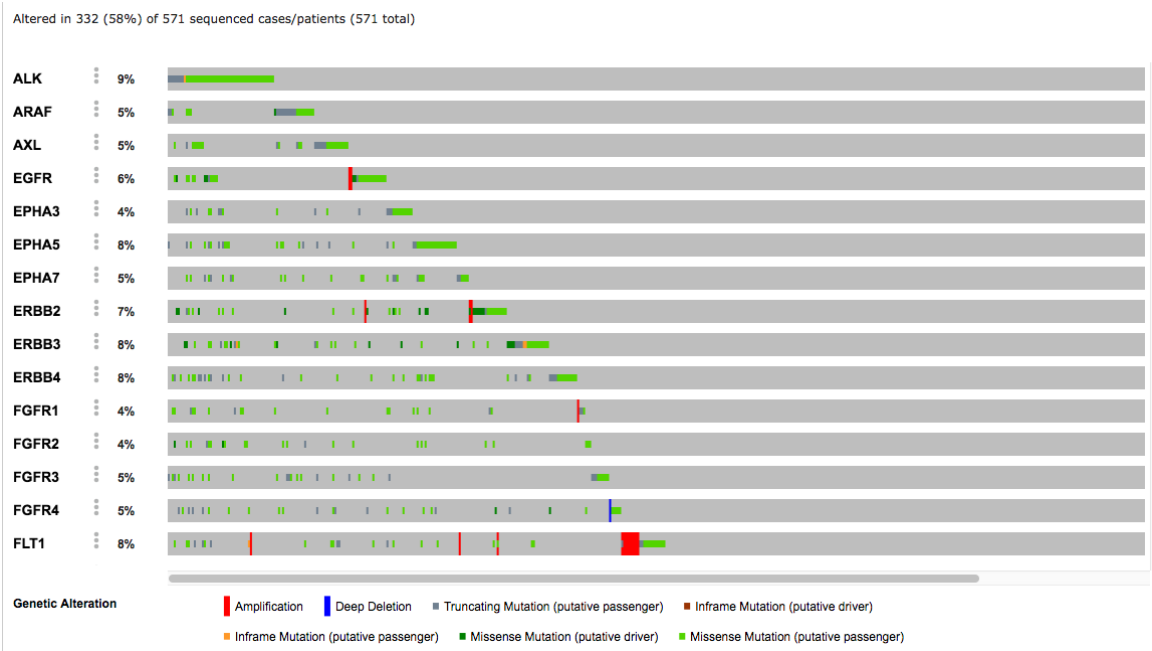


Figure 13. PIK3CA/AKT/MTOR Pathway Genes

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

RTK Pathway Genes Altered in Mutant Cohort



RTK Pathway Genes Altered in Wild Type Cohort

Figure 14. RTK Pathway Genes

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

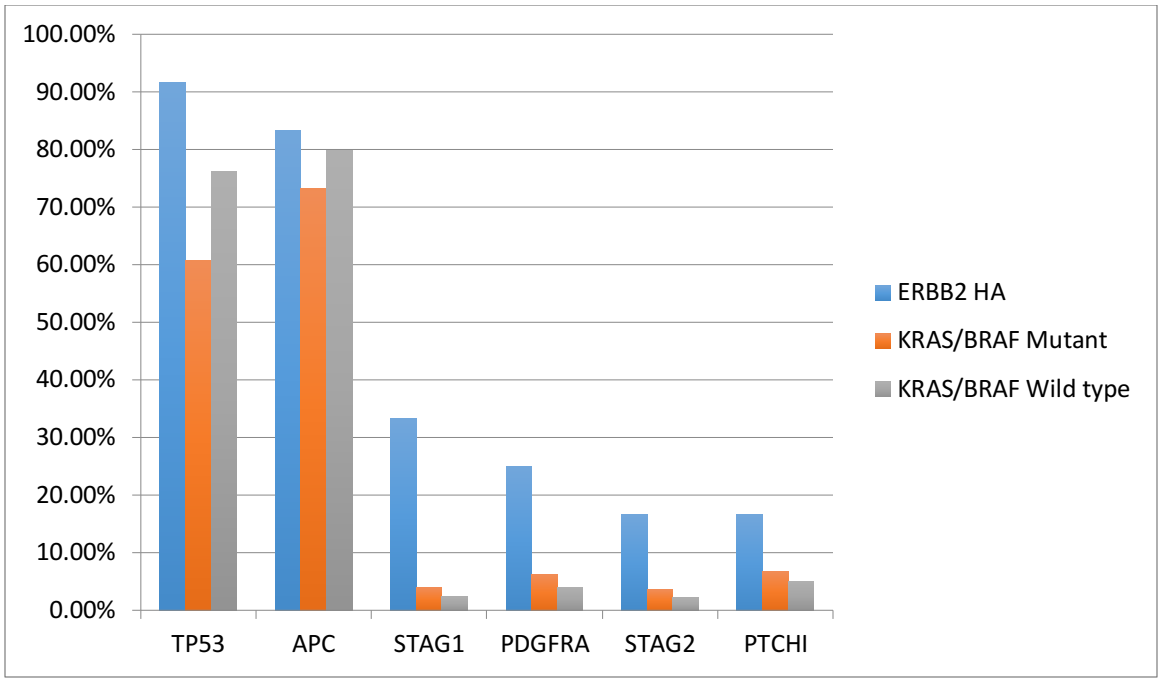
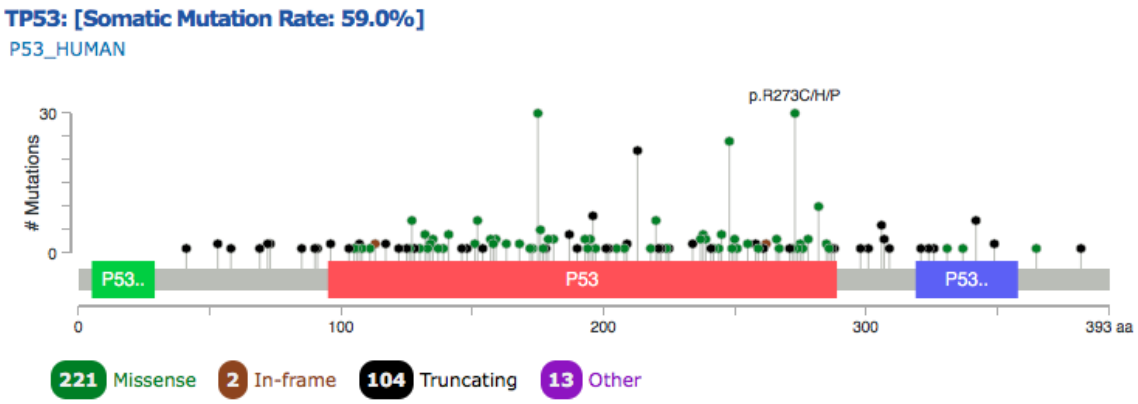


Figure 15. Frequently occurring mutations in cases with *ERBB2* High Amplifications compared to mutant and wild type cohorts

The most common frequently occurring mutations in the 12 *ERBB2* HA patients include *TP53*, *APC*, *STAG1, PDGFRA, STAG2, and PTCHI.* This chart compares the proportion of cases with these mutations in ERBB2 high amp cases, mutant cases, and wild type cases.

Mutant Cohort



Wild Type Cohort

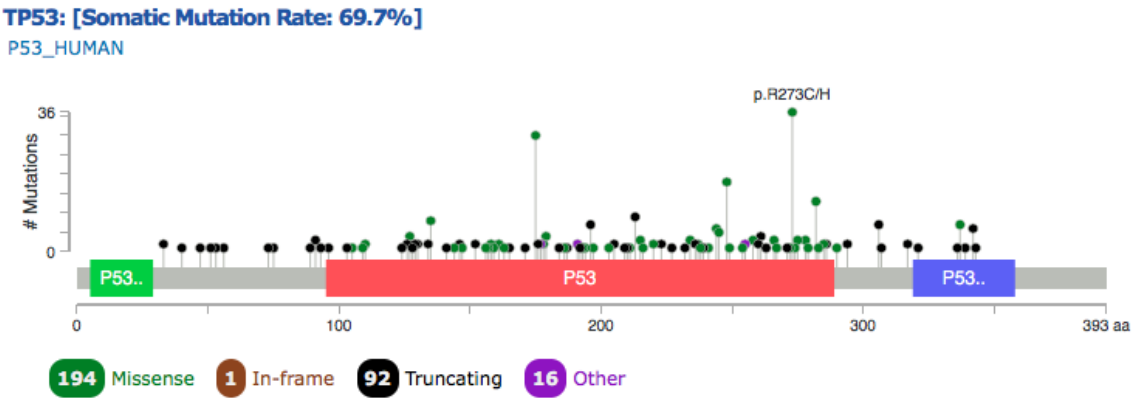


Figure 16. Spectrum of Mutations, *TP53* (Mutant and Wild Type Cohorts)

66% of the *TP53* mutations in the wild type cohort were missense, with most common protein changes at R273C/H/L, R175C/H, and R248Q/W.

http://www.cbioportal.org/
(Gao *et al.* 2013 & Cerami *et al.* 2012)

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