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A Characterization of Colorectal Carcinoma In Patients From The Upper Peninsula of Michigan

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A CHARACTERIZATION OF COLORECTAL CARCINOMA IN PATIENTS FROM THE UPPER PENINSULA OF MICHIGAN

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Abstract

As the third most commonly diagnosed malignancy and second leading cause of cancer-related death, colorectal cancer remains a major global healthcare concern. Despite numerous studies to elucidate the mutations involved in tumorigenesis and assist with the prognostic stratification of patients, individual outcomes and therapeutic responses remain unpredictable. In this study, we performed a retrospective analysis of the clinical and pathological features of colorectal cancers diagnosed in the Upper Peninsula of Michigan. We then characterized the frequency and diversity of six molecular markers (MMR, BRAF, NRAS, KRAS, PIK3CA, PD-L1) in matched samples belonging to 120 patients in our cohort and correlated the findings with cancer registry data.

PCR-based assays were performed to identify point mutations in the RAS, RAF and PIK3CA pathways using zinc formalin-fixed, paraffin-embedded blocks belonging to the patients in our cohort. Additionally, immunohistochemical stains were prepared to assess DNA mismatch repair protein expression and PD-L1 status in the tumor cells. Individual mutations were correlated with the clinical -pathological features of CRC in patients. We noted a higher frequency of primary tumors arising in the proximal colon, as well as a potential prognostic value in KRAS and PIK3CA mutation testing. We believe this is the first population-based study to characterize and correlate mutations with clinicopathological variables in colorectal cancer patients from the Upper Peninsula of Michigan. The findings presented here provide additional insight regarding the tumor

microenvironment at various stages of disease and may lead to more effective patient management strategies as well as the development of new companion diagnostics.

1 Introduction and Literature Review

Although colorectal cancer (CRC) continues to be extensively studied, it remains the third most commonly diagnosed malignancy and second leading cause of cancer-related death in the world [1,2] . In 2019, CRC is projected to account for 8.3% of all new cancer diagnoses and approximately 150,000 new diagnoses this year [2]. The estimated five- year relative survival rate for CRC patients is approximately 64%, however, overall survival rates vary significantly depending on a number of factors, including the histological stage and grade of the tumor at the time of diagnosis, the comorbidities of the patient and the chemosensitiviy of the tumor cells [2, 3].

1.1 Genes and Molecular Pathways Involved in Tumorigenesis

The genes and signaling pathways involved in CRC tumorigenesis have been well documented and include WNT/APC/ β-Catenin, MAPK, PI3K/AKT/ mTOR, TGFβ, and TP53 [4,5,6]. These pathways are responsible for regulating normal cell growth, cellular differentiation, proliferation and survival within the colonic crypts. Additionally, each pathway confers biological properties that maintain the composition of the extracellular matrix. The WNT pathway produces proteins that maintain homeostasis of the stem cell niche within the intestinal epithelium and regulate angiogenesis as well as the remodeling of existing vasculature[6,7, 8]. The MAPK signaling cascade regulates cell migration and apoptosis[9]. The PI3K/ AKT pathway controls cytoskeletal rearrangement, protein translation and cell survival[10].

Phenotypically, there are three molecular pathways involved in colorectal carcinogenesis, including the chromosomal instability pathway (CIN), the CpG Island methylator phenotype (CIMP), and the microsatellite instability pathway (MSI). These pathways have unique characteristics but all result from the accumulation of genetic and epigenetic changes that facilitate the malignant transformation of the colonic epithelium. Additionally, the development of neoplastic precursor lesions, such as adenomas or serrated polyps, proceed the formation of malignant lesions[11,12,13,14].

The CIN pathway is associated with approximately 70% of sporadic CRCs and is characterized by large structural chromosomal changes that may include gains, losses, insertions or deletions and result in aneusomy[8,13]. In CRC, truncating mutations in the Adenomatous Polyposis Coli (APC) tumor suppressor gene result in the activation of the Wnt pathway which initiates tumorigenesis. Dysregulation of Wnt pathway results in chromosomal instability and the acquisition of KRAS mutations as carcinogenesis progresses[8,14,15].

The microsatellite instability pathway results from defects in the DNA mismatch repair (MMR) system and is associated with approximately 15% of sporadic CRC[8,15]. DNA mismatch repair proteins are normally expressed by proliferating cells and correct base substitution mismatches and abnormal insertion-deletion loops arising in repetitive DNA sequences known as microsatellites[16]. Hypermethylation of mismatch repair (MMR) genes results in a loss of function of the MMR machinery and accelerate the accumulation of mutations, especially within repetitive, microsatellite regions[8,15].

Consequently, nucleotide expansions occurring in the exons of genes result in frameshift mutations and mutations in tumor-related genes[14].

Microsatellite instability may also be inherited as a germline mutation via Lynch Syndrome. Individuals with Lynch syndrome inherit a mutant MMR gene and consequently have somatic cells that contain one normal and one non-functioning MMR gene. During tumorigenesis, the normal gene MMR gene may become mutated or epigenetically silenced, resulting in the loss of function of the MMR machinery and acquisition of microsatellite instability in the malignant cells[8,14,15].

The CpG Island Methylator Phenotype (CIMP) pathway is characterized by global hypermethylation of CpG island promoters that result in the epigenetic silencing of MMR proteins and tumor suppressor genes[8,14,15,17]. CIMP is believed to be an underlying factor in MSI, since the latter is often associated with promoter methylation of the MLH1 MMR gene[18]. CIMP tumors tend to be hypermutated, with many demonstrating concomitant BRAF mutations[8,15,18].

1.2 Heterogeneity of Colorectal Cancer

A plethora of research has been performed to elucidate the heterogeneity of CRC, a hallmark feature of this malignancy. CRC tumors are comprised of a highly diverse populations of cells, including malignant differentiated colonic cells, colon cancer stem cells, fibroblasts, immune cells and endothelial cells, each interacting with its neighbors through cell signaling proteins and growth factors in the microenvironment[19]. Mutations are believed to be sequentially acquired as a result of genomic instability and contribute

to the overall genetic diversity of the tumor[20, 21]. The protective tumor microenvironment facilitates tumor development and progression by supporting angiogenesis, epithelial-to-mesenchyme transition and adaptive immunity[22,23].

1.3 The Role of Programmed Cell Death Ligand (PD-L1) in Colorectal Cancer

Programmed cell death ligand 1 (PD-L1) is a transmembrane protein that modulates the immune system by binding to receptors on T-cell lymphocytes and antigen- presenting cells, thereby inhibiting immune responses[24, 25, 32, 33]. PD-L1 may also be located on the surface of malignant cells and tumor-infiltrating immune cells (TIC) within the tumor microenvironment[5]. Increased PD-L1 expression on tumor cells may contribute to T-cell "exhaustion" and suppression of the immune system within the tumor. Increased expression of PD-1 is associated with a poor prognosis in many malignancies, including melanoma, esophageal, gastric, hepatocellular and urothelial carcinomas, and is believed to be associated with tumor invasion in CRC, although this has not been fully elucidated[24, 25, 32, 33].

IN CRC, PD-L1 expression has not been shown to occur in a higher frequency in either gender[26]. Increased PD-L1 expression and BRAF mutations with microsatellite instability have been associated with a poor prognosis. PD-L1 expression analyses might be useful in identifying patients who'd benefit from PD-L1 immunotherapies[27].

1.4 Microsatellite Instability Status

The mechanisms associated with DNA damage have been well-documented in the literature and may occur spontaneously during replication processes or from exposure to various environmental factors including chemicals, radiation, radon and UV light. DNA repair mechanisms maintain the integrity of DNA and mitigate nucleotide errors through a variety of processes, including mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair(NER)[16].

In humans, there are four clinically important DNA mismatch repair proteins (MMR), including MLH1, MSH2, MSH6, and PMS2. In normal repair processes, the MMR proteins form heterodimers (MLH1/PMS2, and MSH2/MSH6, respectively) and excise single nucleotide mismatches and insertion / deletion loops from the DNA strand[16],[28]. Epigenetic changes to the MMR genes, such as hypermethylation of the promoter on the MLH1 gene, result in the loss of expression and consequent dysfunction of the DNA MMR proteins[14,15,28]. Deficiencies or dysfunction of the MMR proteins correlate with microsatellite instability in the tumor [34, 35, 36, 37].

Short segments of repeating nucleotides or microsatellites are located throughout the genome. These repeats are prone to nucleotide mismatch errors arising from polymerase slippage during the replication process[29]. The MMR pathway plays a key role in recognizing and excising errors, as described above. If the MMR proteins are deficient or not functioning properly, alterations occurring during the replication process are not corrected, and result in the accumulation of mutations[14,15,28].

Microsatellite instability (MSI) or MMR dysfunction is noted in approximately 15% of CRC tumors, with MLH1 being the most frequently deficient MMR protein[23]. MSI has both prognostic and therapeutic implications. Primary tumors that have deficient MMR proteins tend to respond to fluoropyrimidine therapy (5-flurouracil) and consequently, confer improved outcomes[23,30]. Patient's whose tumors are MSI may also benefit from immunotherapy[27].

1.5 Mutations in the MAPK Pathway and CRC

The relationship between mutations in the MAPK pathway and the development and progression of cancer have been well documented, with the RAS and RAF oncogenes being the most frequently encountered somatic mutations resulting in cancer[15]. RAS and RAF gain-of-function mutations bypass prerequisite EGFR signaling and independently activate the MAPK pathway[31]. RAS and RAF mutations rarely occur concomitantly, suggesting that these tumorigenic pathways differ and offer no selective advantage for tumors to harbor both[5].

1.6 KRAS

The KRAS oncogene is responsible for activating the MAPK and PIK/AKT/mTOR signaling pathways by transmitting signals received from receptor tyrosine kinase (RTK) to BRAF and PIK3CA, respectively[9, 32, 23]. Point mutations in the KRAS gene activate signaling pathways, independent of growth factor / RTK binding. The KRAS gene is one of the most frequently mutated genes associated with cancer and has been reported in numerous malignancies, including colon cancer,

cholangiocarcinoma, pancreatic and lung cancer [33, 34]. The diversity and frequency of KRAS mutations is a hallmark feature of CRC, with approximately 40% of CRC tumors harboring at least one KRAS mutation[9,34,35]. Anatomically, KRAS mutations are distributed throughout the colon, with females more likely to have a KRAS mutation in transverse and descending colon compared to males[35]. In CRC, KRAS mutation analysis is currently conducted to predict the efficacy of anti-EGFR therapy, however, research is revealing that it may have a prognostic value as well[34,36, 37]. KRAS mutations primarily cluster around mutational hotspots in codons 12 and 13[9,32,35]. KRAS mutation G12V has been associated with more advanced malignancies and confers a poorer prognosis compared to other KRAS mutations[9,38, 34]. Similarly, in recurrent and metastatic colorectal cancer (mCRC), KRAS G13D is associated with poor patient outcomes[32]. KRAS mutations may also be found in codons 61 and 146, with the later noted almost exclusively in CRC[34]. The significance of mutations in codons 61 and 146 has not been fully elucidated, as the recommendation to include these mutations as part of extended mutation analyses in the clinical laboratory was recently made[9,32, 39].

1.7 NRAS

Approximately 2-4% of CRC have NRAS mutations[5,9]. NRAS mutations cluster within codons 12, 13 and 61 and may represent a distinct subtype of CRC, because they demonstrate different clinicopathological characteristics vs those associated with other RAS-family genes[32]. Clinically, NRAS mutations arise in mucinous adenocarcinomas in the distal colon and are demographically associated with older patients. NRAS are often associated with localized disease and confer a better prognosis compared to KRAS[40].

1.8 BRAF

BRAF mutations are associated with a variety of malignancies, including melanoma, papillary thyroid carcinoma, colorectal carcinoma, ovarian and lung cancers[41]. They are identified in 10-15% of CRC tumors and are typically mutually exclusive of KRAS mutations[9,30,32]. The most common BRAF mutation, (V600E), is associated with colon cancer arising in the proximal colon. Phenotypically, BRAF V600E is frequently identified in older, female patients with poorly-differentiated mucinous adenocarcinomas that demonstrate MSI [9,30,32,42,43]. Patients with BRAF mutations typically have a poorer overall survival when compared to patients whose malignancies demonstrate wild-type BRAF[20, 23]. Consequently, BRAF mutation analysis is useful for the prognostic stratification of patients with colorectal cancer and also serves as a biomarker to assist oncologists with predicting patient response to anti-EGFR therapies[44].

1.9 PIK3CA

The PIK3CA gene is responsible for activating the PIK/AKT/mTOR pathway[10,45]. Amino acid substitutions in the p110α protein have been associated with a variety of cancers, including glioblastoma, gastric, head and neck, endometrial, breast, ovary, lung and colorectal cancers[45, 46, 47]. Point mutations in the PIK3CA gene are present in approximatley 10-20% of CRCs and are most frequently clustered in exons 9 and 20[35,47]. PIK3CA mutations are typically identified in poorly differentiated adenocarcinomas and concurrent metastatic liver samples[42] Additionally, PIK3CA mutations present concomitantly with KRAS mutations and have been associated with chemoresistance[42,48].

Patients with PIK3CA / KRAS co-mutations reportedly have poor outcomes, with shorter disease-free survival and high mortality rates[41]. Research suggests that PIK3CA mutations may also be a biomarker to predict response to radiation therapy[45]. Additionally, retrospective studies have suggested that PIK3CA mutation analysis may serve as a predictive marker for patients who'd benefit from adjuvant aspirin therapy, however, further data is needed to make testing for this biomarker a recommendation[39,49].

1.10 Primary Tumor Location and Prognosis

Numerous bodies of work have demonstrated that tumors arising in the proximal or right colon (i.e. cecum, ascending colon, hepatic flexure) have differing embryologic origins, molecular genetic signatures and prognoses compared to those arising in the distal or left colon (i.e. splenic flexure, descending & sigmoid colon) [39,40]. The sidedness of primary tumors has also been shown to be prognostically valuable, however, it would be an over simplification to think of the two "sides" of the colon as completely separate entities, as the prevalence of mutations varies within the anatomic sites on the same side of the colon as well as from cecum to rectum[42].

In general, colon cancer arising in the proximal colon is demographically associated with older, female patients and patients with familial cancers that make them genetically predisposed to CRC[9,42,50]. Histologically, right-sided colon cancers arise from the serrated tumor pathway and tend to be classified as intermediate- to high-grade mucinous, signet-ring or undifferentiated adenocarcinoma at the time of diagnosis[43], [50].

Additionally, they tend to have an advanced American Joint Committee on Cancer (AJCC) pathology stage and greater extent of invasion compared to CRC that arise elsewhere in the colon[51]. Consequently, right-sided cancers are associated with a poor prognosis and a higher prevalence of recurrence and metastasis. When metastasis occurs, right-sided CRC tend to metastasize to regional lymph nodes, the peritoneum and liver [50].

From a mutation characterization perspective, colon cancer arising in the proximal colon tends to demonstrate hypermethylation (CIMP) with a high level of microsatellite instability (MSI-High) [50,52,53,]. KRAS mutations are detected in greater than 50% of tumors arising in the cecum and ascending colon, but this frequency decreases distally across the colon, with the exception being the rectum[9,42]. BRAF V600E mutations are also associated with right-sided colon cancer and generally denote a poor prognosis[52]. Anti-EGFR therapy is not recommended for patients with tumors arising in the proximal colon, because of the high frequency of RAS-family mutations[30]. Instead, anti-VEGF monoclonal antibody therapy may be the adjuant therapy of choice for patients with proximal colon cancers, along with standard cytotoxic agents, such as Folfox (5FU, leucovorin, oxaliplatin) or folfiri (5FU, leucovorin, irinotecan) [41,42].

Colon cancer arising in the distal colon has a favorable prognosis compared to those arising in the proximal colon. Macroscopically, left-sided colon cancers encircle the wall of the colon, and constrict and narrow the lumen as they grow[52]. Consequently, they tend to be less advanced at the time of diagnosis, largely due to the early onset of clinical symptoms (i.e. blood in stool, narrow stool, obstruction) and shorter interval between carcinogenesis and diagnosis[53]. When metastasis occurs, left-sided colon

cancers tend to metastasize to the lungs or bone[50]. Demographically, left-sided colon cancers are more common in males[42]. Mutationally, distal tumors are associated with PIK3CA mutations in the descending and sigmoid colon and KRAS mutations in the rectum[23,42]. Additionally, distal tumors demonstrate chromosomal instability [54]. Patient's with left-sided colon cancer benefit from anti-EGFR monoclonal antibody therapies (i.e. Erbitux) in addition to cytotoxic therapy, i.e. Folfox / Folfiri, provided their tumor has normal, wt-KRAS[32, 42, 55].

1.11 Therapeutic Strategies for CRC

Treatment strategies for CRC is based on the histologic grade and stage of tumors as well as the molecular mutations they harbor. Colorectal cancers are pathologically graded and staged based on standards developed by the World Health Organization (WHO) and the American Joint Committee on Cancer (AJCC)[23,56]. WHO grading categorizes colonic adenocarcinomas based on the morphology of malignant cells as well as their architecture or organization within the tissue. Grade I adenocarcinomas are denoted as "well-differentiated" if the malignant cells microscopically resemble normal colonic cells with uniform, basally located nuclei, and >95% of the malignant cells forming glands[57, 58]. Grade II, moderately differentiated adenocarcinoma is characterized by a loss of nuclear polarity among the malignant cells, and 50-95% of the malignant cells forming glands within the tissue[57, 58]. Grade III, poorly differentiated adenocarcinoma, is characterized by sheets of malignant cells that largely lack glandular architecture[57, 58].

AJCC staging assists clinicians with determining prognosis and treatment options. It standardizes the reporting of the pathological features of tumors, and includes details regarding the depth of tumor invasion within the tissue and the extent of nodal, vascular and distant metastases. AJCC staging uses TNM nomenclature, where T represents the depth the tumor has invaded into the wall of the colon, N denotes the number of lymph nodes containing metastatic tissue and M designates distant site metastasis[56, 63]. Stage I (T1or T2) denotes localized tumors that haven't invaded beyond the muscularis propria in the abdominal wall. Stage II (T3N0, T4N0) denotes tumors that have invaded through the muscularis propria and penetrated the visceral peritoneum but have not invaded the lymph nodes. Stage III tumors invade the lymphatics and represent regional disease. Stage IV malignancies are those with distant metastasis to one or more organs[56, 63].

The National Comprehensive Cancer Care Network provides clinicians with treatment guidelines based on the resectability of the malignancy and AJCC staging information. For patients with localized disease (stage I-II) surgical resection and observation is the standard treatment. Some stage II malignancies (T3, T4), however, may carry a higher-risk for microinvasion, especially if the cells in the tumor were poorly differentiated or if the tumor penetrated the vascular or lymphatic system. For this subset of patients, adjuvant fluoropyrimidine therapy might be administered following surgical resection. Stage III-IV malignancies are often treated with surgical resection and chemotherapy. The specific treatment employed by clinicians is based on the tumor profile and comorbidities of the individual patient[23].

Chemotherapeutic treatment options for patients with CRC typically include 5-fluorouracil (5FU) or its oral prodrug, Capacitibine[59]. Leucovorin, a compound similar to folic acid, is often administered with 5FU to facilitate the binding of 5FU to malignant cells, thereby enhancing its effect[60]. 5FU may also be administered in combination with other cytotoxic drugs, such as oxaliplatin or irinotecan. Additionally, monoclonal antibody therapies that target vasoendothelial growth factor or epidermal growth factor receptor may be prescribed, depending on the mutational status of the patient's tumor[31].

The mutational status of colorectal tumors assists with therapy selection and efficacy. Studies have shown that patients with deficient MMR tumors respond better to 5FU therapy compared to those with proficient DNA repair mechanisms[61, 62]. Additionally, studies have shown that patients with MMR tumors respond to checkpoint inhibitor therapy[27, 62]. The RAS-family (NRAS and KRAS) mutational status assists with identifying patients who'd benefit from anti-EGFR therapy in combination with 5FU. Patients with RAS mutations are ineligible for anti-EGFR therapy[9], [39].

1.12 Colorectal Cancer Mutation Testing

The key societies that provide procedural recommendations to clinical laboratories updated the CRC molecular testing guidelines to standardize mutational analyses and facilitate targeted therapy selection. Specifically, the guidelines recommended that extended NRAS and KRAS mutation analysis be performed to determine the clinical utility of anti-EGFR therapy. Patients with RAS mutations don't benefit from anti-EGFR therapy and consequently, are ineligible for regimens that would

otherwise include it. Further, BRAF V600E mutation analysis and microsatellite instability testing is recommended for prognostic stratification purposes. The societies also considered including PIK3CA mutation panels to their testing guidelines to facilitate the identification of patients who might respond to aspirin therapy, but there was insufficient evidence to formally make this recommendation. The authors noted, however, that there is a the need for additional research[39]

1.13 Specific Aims of Work

As the literature review supports, although CRC has been extensively studied, the prognostic and therapeutic role that individual mutations and co-mutational pathways play in individual chemotherapeutic response has not been fully elucidated. In this population-based study, we analyzed the clinicopathological features of a retrospective colorectal cancer patient cohort over a seven-year span of time. In chapter two, our specific aims were to examine (1) the relationships between specific clinicopathological variables and (2) identify variables that might facilitate the management and prognostic stratification of CRC patients.

In chapter three, our specific aims were to examine (1) the frequency and diversity of specific mutations, 2) determine the relationships between the mutations and clinicopathological variables, and 3) elucidate the tumor microenvironment at various stages of disease to potentially facilitate the development of new companion diagnostics and more effective patient management strategies.

2 A Retrospective Population-based Study of Colorectal Cancer in the Upper Peninsula of Michigan

Cancer is the second leading cause of death for Michigan residents, with cardiovascular disease being the first[53]. The incidence rate for colorectal cancer is approximately 4%, with a reported 36.3 individuals per 100,000 diagnosed with colon or rectal cancer each year[63]. This year, approximately 5,000 Michigan residents are expected to be diagnosed with CRC and an estimated 1,650 are expected to pass away as a result[63]. Similarly, according to the Michigan Cancer Surveillance Program, in the Upper Peninsula of Michigan, an average of 36 residents per /100,000 were diagnosed with CRC between 2012-2016 (Figure A.1). Fifty-eight percent of the malignancies had regional or distant metastatic disease at the time of diagnosis and an average of 13.3 residents per 100,000 passed away as a result (Figure A.2)[64].

Risk factors for CRC have been well-documented and include genetic, environmental and lifestyle-associated factors[65], [66]. Colon cancer typically affects people who are over the age of 50, but first-degree relatives with a history of CRC, or a personal history of colon polyps or ulcerative colitis increase risk and are important considerations when determining the appropriate age to initiate CRC screening [67]. Lifestyle choices that contribute to an increased risk of developing colon cancer, including being sedentary, overweight or obese, consuming a high fat, low fiber diet with a high red meat content, and high-risk behaviors, like heavy alcohol and tobacco use[7, 68, 3].

Michigan is ranked in the top ten states with a high prevalence of obesity, with 1 in every 10 adults having a BMI of >30 (obese) and 35% of residents being overweight (with a BMI between 25-29.9)[64,69]. In the Upper Peninsula of Michigan, 67% of the residents have a BMI that would rank them as either overweight or obese[64,69]. Additionally, Michigan residents report the following lifestyle choices that potentially increase risk of cancer: cigarette smoking (21%), alcohol use (16%), physically inactive lifestyle (25%)[64]. Fortunately, seventy percent of respondents also reported that they follow the recommended CRC screening guidelines[64].

The aim of this work was to examine (1) the relationships between specific clinicopathological variables in CRC patients and (2) identify variables that might facilitate the management and prognostic stratification of CRC patients.

2.1 Materials and Methods

2.1.1 Study Design and Patient Population

This retrospective, population-based study was based on a cohort of 541 patients who underwent surgical resection for primary, recurrent or metastatic colorectal cancer (CRC) between the years of 2004-2007 and 2013-2015 in a rural healthcare system in Michigan's Upper Peninsula. Patient consent forms were obtained according to institutional policies. Correlative, anonymized patient demographic and clinical - pathological information was obtained from the Colon Cancer Tumor Registry following approval from the Institutional Review Board.

From this cohort, the age, gender, specific anatomic location of the primary malignancy, histological grade and AJCC stage of tumor, gastroenterologists procedural

notes, surgical / chemotherapeutic / radiological treatment information and vital status was obtained.

2.1.2 Inclusion and Exclusion Criteria

This study included patients who underwent surgical resection for CRC and were ≥18 years of age, with a diagnosis of primary, recurrent or metastatic adenocarcinoma of any histologic grade and AJCC stage (Tis-T4). Carcinomas, neuroendocrine carcinomas, carcinoid tumors and lymphomas that were surgically excised from the colon were also included in this retrospective study.

2.1.3 Histological Classification of Colon Cancer

Tumors belonging to this cohort of patients were categorized based on the World Health Organization's (WHO) histological grading and American Joint Committee on Cancer (AJCC), 7^{th} edition, staging as denoted in the cancer registry entries.

2.2 Results

2.2.1 Patient Demographics

Of the 541 cancer registry entries analyzed in this study, 56% (303) belonged to male patients and 44% (238) belonged to females. The average age of the male subjects in this study was 66 years of age, with a range of 29 to 93 years of age. The average age of the female subjects in this study was 70 years of age, with a range of 19 to 96 years of age (see Table A.1).

2.2.2 Distribution of Primary Tumors

The primary malignancies in this study arose in the following anatomic locations: 14% cecum, 12% ascending colon, 2% hepatic flexure, 11% transverse colon, 2% splenic

flexure, 2% descending colon, 18% sigmoid colon, and 22% rectum. Additionally, some of the samples in the cohort had the following "alternative" site designations: 5% right colon, 6% colon, 0.5% overlapping lesion, 1% left colon, 1% ileocecal valve, 1% appendix, and 0.2% anal-rectal junction (see Figure 2.3). In terms of proximal vs distal colon, the primary cancers had the following distribution pattern: 34% proximal colon (cecum, ascending colon, hepatic flexure), 11% transverse colon, 24% distal colon (splenic flexure, descending colon, sigmoid colon), 23% rectum, 1% appendix, 6% colon, NOS, 0.7% ileocecal valve, 0.3% overlapping lesion, NOS. The distribution of malignancies by anatomic site and patient demographics may be found in Figure A.4 and Table A.1.

2.2.3 Histologic Grading and Staging by Tumor Site

A majority of the tumors in our study were histological grade 2 adenocarcinomas, however, some of the tumors were only graded as "adenocarcinoma". Additionally, gastrointestinal carcinoid tumors, squamous cell carcinomas, lymphomas, and goblet cell tumors were included in this study.

The AJCC staging for the samples in this study were as follows: Stage 0 (1%; 4/541), Stage I (20%, 109/541), Stage II (26%; 144/541), Stage III (26%; 142/541), Stage IV (12%; 62/541) and "unable to stage / stage x" (14%; 77 / 541). AJCC staging wasn't applicable for three non-colonic malignancies (i.e. lymphoma, Burkitt's Lymphoma, and Squamous Cell Carcinoma). Approximately 30% of the malignancies arising in the ileocecal valve, cecum, ascending colon, and descending colon were AJCC Stage III malignancies while those arising in the hepatic flexure, transverse colon, and splenic flexure were primarily stage II malignancies; sigmoid colon and rectum were Stage I –II.

In total, 48% of the patients in the cohort had localized disease (Stage I-II), with 26% having regional disease (Stage III) and 11% having distant metastatic disease (see Figure A.4).

2.2.4 Clinical Rationale for Colonoscopy Referral

Based on data obtained from the gastroenterologist's procedural notes, forty-eight percent of the patients in this cohort had colonoscopies that were classified as "diagnostic" because they were experiencing classical clinical symptoms of colon cancer including blood in stool, rectal bleeding and/or positive fecal occult blood test results or had polyps &/or suspicious lesions discovered during the colonoscopy procedure. Unfortunately, the procedural notes indicated that the reason for the original referral was unknown in 20% of the patients in the cohort and 16% had colonoscopies performed without any additional ancillary text notes. Only 3% of the patients in the study had colonoscopies that were designated as "screening" and, interestingly, one patient had their cancer detected via virtual colonoscopy (see Table A.2).

2.2.5 Treatment

Treatment administered to this cohort consisted of the following: surgical intervention and observation (48%), Surgical and chemotherapeutic treatment (33%), surgical, chemotherapeutic and radiotherapy (18%), surgical intervention and radiotherapy (0.6%), patient declined chemotherapy (0.6%).

2.2.6 Vital Status

Forty-three percent (232/541) of the patients in our cohort had expired at the time the data was extracted from the system. From a demographic standpoint, 57% (132/232) of the patients who expired were males and 43% (100/232) were females. The primary

tumor location for patients who expired were as follows: 32% proximal colon (75/232), 22% distal colon (51/232), 11% transverse colon (26/232), 21% rectum (49/232), 12% colon (27/232) and 2% app (4/232). The vital status for this cohort is non-informative, as the cause of death was not specified in the registry data.

2.3 Discussion

To our knowledge, this is the first study to characterize the clinicopathological attributes of colon cancer in patients from the Upper Peninsula of Michigan. In our cohort, there was a slightly higher percentage of male patients (56%) compared to female patients (44%), but this is consistent with national demographic data and correlates with the fact that 22% of the malignancies in our cohort arose in the rectum[3]. The average age of male vs. female patients treated for CRC in Upper Michigan was 66 and 70 years of age, respectively, and is approximately 2 years younger than the national statistics[3]. Our cohort had a higher incidence of primary tumors arising in the proximal colon (34%) compared to those arising in the distal colon (24%). Interestingly, the incidence of proximal colon cancer was nearly 1.5x higher than the national average of 20%. This finding was not artificially increased by the number of females with malignancies in the right colon, because the male-to-female ratio was similar (i.e. 46% males vs 54% females).

Histologically, a majority of the colon cancers were histological grade 2. From an AJCC staging standpoint, the colon cancers in our cohort had a favorable staging distribution compared to national data, with nearly half of the patients (46%) having tumors that were localized (grades 0-II)[56]. This may be evidence of the successful

employment of early CRC screening programs and supports the Michigan Cancer Surveillance data in which 70% of Upper Michigan residents indicated they comply with CRC screening guidelines[64]. Additionally, only twenty-six percent of the patients in our study had regional disease and 12% had distant metastatic disease. Consistent with national trends, malignancies in the proximal colon (i.e. ileocecal valve, cecum, ascending colon) demonstrated more advanced disease, whereas those arising in the hepatic flexure and throughout the distal colon represented localized disease. The descending colon was the exception with 36% of the tumors being stage III. This largely supports the theory that, because of clinical symptoms, CRC in the distal colon is discovered and resected at earlier stages vs. those arising in the proximal colon.

We also noted that a majority of the gastroenterologists' procedural notes suggested that patients were referred for colonoscopies because of clinical symptoms associated with colorectal cancer. Additionally, we noted that 53% of patients had suspicious lesions or polyps identified during their colonoscopic procedure. The latter is counterintuitive to the CRC staging that we identified in our samples and is disconcerting as well. Access to routine healthcare and individual beliefs regarding preventative practices may present educational opportunities for the perusal of Upper Peninsula public health facilities.

2.4 Conclusion

This study provided a thorough clinicopathological picture of colorectal carcinoma in Michigan's Upper Peninsula. Knowing that patients in the UP have 1.5x the incidence of CRC arising in the proximal colon provides primary care physicians with

the opportunity to encourage regular screening colonoscopies vs. other screening modalities that may not interrogate the proximal colon. This study also emphasized that our population is high-risk for CRC, both from a body mass index standpoint, self-reported alcohol and tobacco product use, as supported by the prevalence of diagnostic colonoscopies.

2.5 Future Opportunities

The discovery of the predilection of proximal colon cancer in patients of rural Upper Michigan presents several opportunities for future initiatives. Firstly, it is clinically relevant knowledge that may benefit public health clinicians and primary care physicians by affording them the opportunity to development strategies to aid in the early detection of proximal CRC malignancies. Secondly, the knowledge gained from this study may benefit gastroenterologists and surgeons by alerting them to the need to interrogate the proximal colon when applicable. Thirdly, the findings of this work and the national increased incidence of CRC arising in younger adults merit the continued surveillance of the clinicopathologic features of CRC in Michigan's Upper Peninsula.

3 Colorectal Cancer in the Upper Peninsula of Michigan: A Population-based Study characterizing molecular mutations and clinical attributes

An enormous amount of money and time has been invested in researching colon cancer, and yet the heterogeneity of the disease renders us unable to identify a biomarker to effectively diagnose and treat this insidious disease. Innumerable articles and clinical trials speak to the need for additional insight into tumor evolution and the prognostic role that the primary tumor's anatomic location plays in therapeutic response[36,42,70,71]. In this chapter, our specific aims were to (1) examine the frequency and diversity of specific mutations in our cohort, 2) determine the relationships between the mutations and clinicopathological variables, and 3) elucidate the tumor microenvironment at various stages of disease to potentially facilitate the development of new companion diagnostics and more effective patient management strategies.

3.1 Materials and Methods

3.1.1 Study Design and Patient Population

A retrospective analysis was performed on 120 patients who underwent surgical resection for primary, recurrent or metastatic colorectal cancer between the years 2004-2007 within a health network in Michigan's rural Upper Peninsula. Patient consent forms were obtained according to institutional policies. Correlative, anonymized patient demographic and clinical- pathological information was obtained from the Colon Cancer Registry following approval from the Institutional Review Board.

In total, approximately two-thousand, five hundred zinc-formalin-fixed, paraffin-embedded tissue blocks belonging to the patient cohort were retrieved and 230 blocks were selected for subsequent analysis. These "matched" samples represented various stages of disease (AJCC stage Tis-T4) and included biopsies, colon polyps with high-grade dysplasia / adenocarcinoma in-situ, resections of primary tumors, and resections with corresponding metastatic tissue. Carcinomas, neuroendocrine carcinomas, carcinoid tumors and lymphomas excised from the colon were excluded from the analysis.

The zinc-formalin-fixed, paraffin-embedded tissue samples were analyzed to identify the presence of 73 possible point mutations in the KRAS, BRAF/NRAS, and PIK3CA genes. Additionally, immunohistochemical stains (i.e. MLH1/PMS2, MSH2/MSH6) were performed to characterize the functionality of the DNA mismatch repair system and PD-L1 expression (adaptive immunity) in the tumor cells.

3.1.2 Tissue Selection and DNA extraction

Hemotoxylin and Eosin-stained slides were retrieved and reviewed by the PI and a pathologist to confirm the diagnosis, histologic grade and staging of each sample. Optimal blocks, defined as those with ≥10% tumor content, were selected for subsequent mutation and IHC analysis.

DNA was manually extracted from the archived tissue blocks using the Cobas® DNA Sample Preparation Kit (Roche, Indianapolis, IN). Specifically, a five- μm section was obtained from each tissue block and placed in a 1.5ml PCR-safe tube, using protocols previously described to avoid DNA contamination[72]. Next, the section was deparaffinized in xylene and rehydrated in 100% ETOH. The tissue was lysed via a

protease and passed through a filter column to sequester the DNA and remove impurities. The nucleic acids were eluted from the filter and the genomic DNA concentration was determined via a Nanodrop spectrophotometer. The concentration of the stock DNA in each sample was diluted to 2ng/ul, using a dilution calculation provided by the manufacturer, to standardize DNA content in the samples prior to the amplification and mutation detection. Stock samples were stored in the -20^{0}C freezer until use.

Also per the manufacturer's specifications, samples initially yielding invalid mutation test results were retested after new dilutions of the stock DNA were prepared. If invalid results were acquired a second time, fresh DNA was extracted from a new 5µm section of FFPET tissue.

3.1.3 K-RAS Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) Mutation Analysis

"Extended" KRAS mutation analysis was performed using a "life science, research only" (Roche Diagnostics, Indianapolis, IN). The assay utilized real-time PCR, specific base-pair primers and fluorescence resonance energy transfer (FRET) probes to detect mutations in the following targeted KRAS regions: KRAS Exon 2, codons 12 and 13 (G12A, G12C, G12D, G12R, G12S, G12V, G13A, G13C, G13D, G13R, G13S, G13V), KRAS Exon 3, codons 59 and 61 (A59E, A59G, A59S, A59T, Q61E, Q61Hc, Q61Ht, Q61K, Q61L, Q61P, Q61R), KRAS Exon 4, codons 117 and 146 (K117Nc, K117Nt, A146P, A146T, A146V). A mutant control, a process control and a negative control were incorporated into each run to confirm the validity of the run. Following the completion of the RT-PCR reaction, all data files were uploaded into the Roche web tool (http://oncologyresearchkits.roche.com/data-analysis) for analysis.

3.1.4 B-RAF Proto-oncogene (BRAF) and Neuroblastoma RAS Viral Oncogene Homolog (NRAS) Mutation Analysis

"Extended" BRAF and NRAS mutation analysis was performed using a "life science, research only" assay developed by Roche Diagnostics (P/N: 07659962001, Roche Diagnostics, Indianapolis, IN). The assay utilized real-time PCR, specific base-pair primers and fluorescence resonance energy transfer (FRET) probes to detect the following mutations: BRAF Exon 11 (G466A, G466V, G469A, G469R, G469V), BRAF Exon 15 (V600E, V600E2, V600D, V600K, V600R, K601E), NRAS Exon 2 (G12A, G12C, G12D, G12R, G12S, G12V, G13A, G13C, G13D, G13R, G13S, G13V, A18T), NRAS Exon 3 (Q61Ht, Q61Hc, Q61K, Q61L, Q61P, Q61R), and "other" NRAS Exon 3and 4mutations, including A59D, K117Nc, K117Nt, A146T, A146V. A mutant control, a process control and a negative control were incorporated into each run to confirm the validity of the run. Following the completion of the RT-PCR reaction, all data files were uploaded into the Roche web tool (http://oncologyresearchkits.roche.com/data-analysis) for analysis.

3.1.5 Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha (PIK3CA) Mutation Analysis

"Extended" PIK3CA mutation analysis was performed using a "life science, research only" assay (Roche Diagnostics, Indianapolis, IN). The assay utilized real-time PCR, specific base-pair primers and fluorescence resonance energy transfer (FRET) probes to detect the following mutations or targeted regions: PIK3CA Exon 1 (R88Q), PIK3CA Exon 4 (N345K), PIK3CA Exon 7 (C420R), PIK3CA Exon 9 (E542K, E545A, E545D, E545G, E545K, Q546E, Q546K, Q546L, Q546R), PIK3CA Exon 20 H1047L,

H1047R, H1047Y, G1049R). Following the completion of the RT-PCR reaction, the mutation analysis was performed by the Cobas 480z analyzer. A mutant control, a process control and a negative control were incorporated into each run to confirm the validity of the run.

3.1.6 Immunohistochemical Assessment of DNA Mismatch Repair Proteins

A series of immunohistochemical stains were performed using the Benchmark Ultra System (Roche Ventana, Tucson, Arizona) to assess the presence or absence of nuclear expression in neoplastic cells for four DNA mismatch repair proteins, MLH1/PMS2 and MSH2/MSH6. Four serial tissue sections (4 μm) were collected from each FFPET block and mounted on positively-charged microscope slides. The first slide was stained with anti-MLH1 (clone M1) (Roche Ventana, Tucson, Arizona); the second slide was stained with anti-MSH2(clone G219-1129) (Roche / Ventana, Tucson, Arizona); the third slide was stained with anti-MSH6 (clone 44) (Roche / Ventana) and the fourth slide was stained with anti-PMS2(clone EPR3947) (Roche / Ventana, Tucson, Arizona). "Pre-qualified" colon cancer tissue (i.e. colon cancer tissue that previously demonstrated intact MMR proteins) served as the positive control tissue. All slides were independently reviewed and scored by both the PI and a qualified pathologist to determine the mismatch repair protein status.

3.1.7 Immunohistochemical Assessment of PD-L1 Expression

Immunohistochemical assays were performed on the Benchmark Ultra (Roche/Ventana, Tucson, AZ) using anti-PDL-1 antibody (clone SP263)

(Roche/Ventana, Tucson, AZ) to evaluate PDL-1 membranous expression in tumor cells in cohort tissue samples. Specifically, three (4 μm) serial sections of tissue were mounted on positively charged glass slides. Hematoxylin & Eosin staining was performed on the first slide to confirm specimen adequacy (i.e. each section contained >50 viable tumor cells with associated stroma, per manufacturer guidelines). If deemed adequate, the second slide containing patient tissue was stained with PDL-1 (clone SP263) and the third with a Rabbit Monoclonal Negative Reagent Control. Human term placental tissue was used for the positive control tissue.

PDL-1 (SP263) was independently quantified by the PI and a qualified pathologist, using investigator-developed scoring criteria to facilitate reproducibility. Specifically, PDL-1 stained malignant tissue was methodically evaluated and the aggregation method was utilized to score percent positivity of membranous staining in viable tumor cells, as follows: 0-<1%, 1-9%, 10-29%, 30-49%, 50-69%, 70-89%, 90-100%. Staining of tumor infiltrating immune cells (IC) served as an internal control and was qualitatively noted but not quantified.

3.2 Statistical Analysis

While much of the analyses in this study were performed using descriptive statistics, R 3.5.3 software (https://www.r-project.org/) was utilized to assess the association between mutational status and various clinical-pathological parameters.

3.3 Results

3.3.1 Patient Demographics

Of the 120 cancer registry entries analyzed in this study, 58% belonged to male patients and 42% belonged to females. The average age of the male subjects in this cohort was 66 years of age, with a range of 33-89 years of age. The average age of the female subjects in this study was 73 years of age, with a range of 45 - 90 years of age.

3.3.2 Distribution of Primary Tumors by Anatomic Site

The primary malignancies in this study were distributed across the anatomic sites of the colon, as follows: 13% cecum, 14% ascending colon, 5% hepatic flexure, 12% transverse colon, 4% splenic flexure, 2% descending colon, 19% sigmoid colon, 18% rectum (Figure A.5). Additionally, some of the samples in the cohort had "alternative" site designations, including: 6% right colon, 4% colon, 2% ileocecal valve, 1% overlapping lesion, 2% left colon. From a proximal vs distal standpoint, the distribution of primary malignancies was as follows: 42% (51/120) proximal colon, 12% (14/120) transverse colon, 23% (27/120) distal colon, 18% rectum, 1% (1/120) appendix, and 4% (5/120) colon, NOS (Figure A.6).

3.3.3 Histologic Grading and Staging by Anatomic Site

The histological grade for the samples in our cohort were as follows: grade 0 (2%), grade 1 (2%), grade 2(73%), grade 3(19%) and grade 4 (4%). The AJCC staging for the samples in this study were as follows: Stage I (29%), Stage II (35%), Stage III (30%), Stage IV (6%) and "unable to stage / stage x" (0.8%). A majority of the

malignancies arising in the ileocecal valve, cecum, sigmoid and rectum were AJCC Stage III malignancies while those arising in the ascending and transverse colon flexure, splenic and hepatic flexures were Stage II.

3.3.4 KRAS Mutation Status by Tumor Location

KRAS mutations were identified in 36% of the patients in our study, with 84% of the mutations arising in exon 2. Thirty-five percent of the samples with a KRAS mutation arose in the proximal colon (p=0.04). Thirty-four percent of the malignancies with a KRAS mutation were AJCC stage I, and the mutations showed a similar distribution pattern across histological grades I-III. Interestingly, 23% of the patients with a KRAS mutation had co-occurring mutations, with 80% of these being PIK3CA point mutations.

Point mutation G12x accounted for 63% of the KRAS mutations identified in our cohort (Table A.4). Malignancies with this mutation were anatomically distributed across the colon, and were primarily histologic grade 2, AJCC stage I-II tumors. Interestingly, 26% of the patients with the G12x mutation had metastatic disease, 7% had synchronous malignancies and 4% experienced recurrence. Additionally, 22% of the patients with KRAS G12x mutation had concomitant mutations, with 27% of these being PIK3CA co-mutations (E545x, H1047x, Q546x). One patient had a BRAFV600E / KRAS G12x co-mutation, which reportedly occurs in 0.001% of CRC tumors [47],[15].

Twenty-one percent of the patients with a KRAS mutation had a G13x point mutation in Exon 2 (Table A.5). Thirty-three percent of these had metastatic colon

cancer, and another patient with this mutation experienced recurrence in a different location in her colon two years after the diagnosis and treatment of her original malignancy. The tumor cells in this patient's original resection demonstrated PD-L1 expression and, while PD-L1 expression was not noted in subsequent specimens, the KRAS G13x mutation was. All of the samples with the KRAS G13x mutation were histological grade 2, with 54% being AJCC Stage III. Of these samples, 64% arose in distal colon.

Mutations in exons 4 and 3 comprised 9% and 5% of the KRAS mutations in our cohort, respectively. The KRAS A146x mutation was detected in two patients, and each had concomitant PIK3CA mutations as well. Two patients also had the KRAS K117x mutation identified in their samples. Both of these patients had metastatic CRC, with malignancies arising in the distal colon. The KRAS Q61x was identified in one patient in our cohort. This patient also had a co-occurring PIK3CA mutation, C420R. Finally, one patient had the A59x mutation detected in a grade 3 adenocarcinoma collected from the sigmoid colon (Table A.5).

3.3.5 BRAF Mutation Status by Anatomic Site

The BRAF V600E mutation was identified in approximately 18% of the patients in our cohort. BRAF V600E mutation was associated with females (p=.001), with seventy-six percent of the patients with a BRAF mutation being female and twenty-four percent male. Fifty-four percent of the malignancies with the BRAF V600E mutation arose in the proximal colon (cecum, ascending, and "right colon, NOS). Eighteen percent of the BRAF V600E point mutations arose in the transverse colon, with nine percent

arising in the splenic flexure and sigmoid colon, respectively. From a grading and staging standpoint, a majority of the tumors with BRAF mutations were histologic grade II or III and represented localized disease (Table A.6).

3.3.6 NRAS Mutation Status by Anatomic Site

Only 6% of the patients in our study had NRAS mutations detected in their tumor samples, with two patients having metastatic CRC and one chemoresistant cancer. The average age of patients with this mutation was 67 years old, with a range of 51-84 years. Seventy-one percent of the NRAS mutations were in exon 3 (Q61x). Forty-three percent of the NRAS mutations arose in the proximal colon, and overall, the specimens with NRAS mutations were histological grade II, AJCC stage I (Table A.7). No concomitant mutations were identified in the patients with NRAS mutations.

3.3.7 PIK3CA Mutation Status by Anatomic Site

Sixteen percent of the patients in our study had a PIK3CA mutation detected in their tumor, with a majority of these arising in exons 9 and 20. Patients with a PIK3CA mutation were, on average, 68 years of age. Twenty one percent of the patients with a PIK3CA mutation had metastatic disease and sixteen percent had synchronous malignancies. Overall, fifty-three percent of the samples with a PIK3CA mutation had one additional co-occurring mutation, and 10% had two concomitant mutations (Table A.8). Interestingly, two patients in our study had co-occurring PIK3CA mutations with an additional BRAF V600E or KRAS G12x mutation. One of the individuals was initially treated for a synchronous malignancy and two years later, experienced recurrent

CRC. More than half of the malignancies harboring PIK3CA mutations arose in the proximal colon and were categorized as histological grade 2, AJCC Stage II lesions.

3.3.8 Concomitant Mutations by Anatomic Site

Twenty-nine percent of the patients in our cohort had multiple mutations identified by IHC or PCR-based mutation analyses (Table A.9). Of these, 43% had primary tumors arising in the proximal colon, with ascending colon having the greatest number of co-mutations. Additionally, the frequency of concomitant mutations gradually decreased from the transverse colon (11%) to the rectum (3%).

3.3.9 Immunohistochemical Analysis of DNA Mismatch Repair Proteins

Eighty percent of the patients in our study had malignancies with intact MMR proteins, while 17% demonstrated a loss of two repair proteins and 3% showed the loss of expression of one DNA repair protein (Figures A.7-A.10 and Tables A.10 – A.12). Of the patients with intact MMR proteins, 31% had metastatic disease. Sixty-one percent of the tumors with intact MMR proteins belonged to males with primary malignancies predominantly arising in the sigmoid colon and rectum. Conversely, a majority of the samples demonstrating the loss of two MMR proteins primarily belonged to female patients (58%) with malignancies arising in the proximal colon. As may be expected, BRAF V600E mutation was often associated with deficient MMR protein expression ($p=2.2\text{^}10^{-5}$)

3.3.10 Immunohistochemical Analysis of PD-L1 Expression

Ten percent of the patients in our cohort had measurable PD-L1 expression levels in their tumor cells, however, expression varied among the matched patient samples (Figures A.11-A.12). In general, PD-L1 expression was observed more frequently in tumors arising in the sigmoid and "right colon", followed closely by those in the rectum, ascending colon and cecum. PD-L1 expression levels of >30% were observed in only 6% of our cohort, and was noted in patients with metastatic disease whose tumors arose in the cecum. Tumors exhibiting 10-29% PD-L1 expression was noted in a patient whose malignancy arose in the appendix and in another patient who had a synchronous malignancy and later experienced recurrent adenocarcinoma in the proximal colon (Table A.13).

3.3.11 Characterization of Synchronous Malignancies

Six patients (3 males, 3 females) in the cohort had synchronous malignancies (Table A.14). The average age of the patients with synchronous malignancies was 78 years of age for the males and 74 for the females. The anatomic distribution of the malignancies was as follows: 8% ileocecal, 17% cecum, 25% ascending colon, 8% transverse, 17% descending colon and 25% sigmoid colon. Sixty-seven percent of the synchronous malignancies were histological grade II and 50% were classified as AJCC stage I tumors.

Interestingly, sixty-seven percent of the synchronous malignancies had intact MMR proteins and 50% had identical mutations within the paired samples. No mutations were identified in four of the synchronous malignancies arising in the ileocecal valve,

sigmoid colon, and cecum. Paired synchronous malignancies arising in the ascending and transverse colon demonstrated a loss of the MLH1/PMS2 MMR proteins and the BRAF V600E mutation.

Paired malignancies with divergent mutations included two samples from the descending colon, where one sample demonstrated <1% PD-L1 expression and a KRAS G12x mutation, while the other only demonstrated a PIK3CA E545x mutation. Additionally, one synchronous malignancy originating in the sigmoid colon showed a KRAS G12x mutation while the other tumor did not. Finally, one synchronous malignancy originating in the ascending colon demonstrated a loss of both MLH1/PMS2 MMR proteins, while the other only displayed a loss of MLH1. The PIK3CA E454x mutation was identified in both of these paired malignancies.

3.3.12 Characterization of Recurring Malignancies

Three female patients in our study experienced recurrent colon cancer within two years post-surgical excision of their primary malignancy. Another patient initially had synchronous malignancies and later developed recurrent cancer. Fifty percent of these tumors arose in the rectum, with the remaining malignancies arising in the "right colon, NOS" (38%) and sigmoid colon (12%). Fifty percent of the tumors were histologically a grade 2, with a majority being AJCC stage II (Table A.15).

Interestingly, the mutations in the recurrent malignancies largely resembled those in the original primary cancer. For instance, for one patient, the KRAS G12x mutation was identified in both the primary and recurring malignancy arising in the rectum.

Another patient had synchronous tumors arising in the "right colon" that exhibited a loss of both MLH/PMS2 MMR proteins, the BRAF V600E mutation and PIK3CA mutations H1047x and E545x. Additionally, both demonstrated PD-L1 expression in the tumor cells, with 10-29% expression in one and 1-9% expression in the other. Two years later, the recurrent cancer in the biopsy and resection demonstrated a similar loss of MMR proteins, PD-L1 expression levels and BRAF V600E mutation. The PIK3CA mutations, however, were not detected in the recurrent malignancy. Lastly, a malignancy that arose in the sigmoid colon displayed 1-9% expression of PD-L1 and the KRAS G13x mutation. The recurrent cancers in the rectum did not express PD-L1 but the KRAS G13x mutation was identified in each.

3.3.13 Characterization of Chemoresistant Malignancies

Approximately 7% (8/120) patients in this study had malignancies that were presumed to be chemoresistent, based on multiple courses of cytotoxic therapy administered over an extended amount of time and treatment notes (Table A.16). Chemoresistence was noted evenly between males and females in this category (4/8 or 50% each). The average age for the males in this category was 51 years of age (range from 46-53 years of age) and 62 for females, with a range of 45-70 years of age. The anatomic location of primary malignancies was as follows: 25% (2/8) rectum, 12.5% (1/8) for each of the following sources: Overlapping lesion, Ascending, Transverse, Sigmoid, Colon, and Right colon. Mutations characterized in this subset of patients / samples included the following: KRAS G13x was detected in one patient's sample (sigmoid), KRAS G12x was detected in one patient's sample (transverse), KRAS 146x

and PIK3CA E545x was detected in one patient sample (ascending colon), NRAS Q61x and MMR repair protein markers (loss of PMS2) was detected in one patient sample (right colon). Interestingly, we were unable to identify mutations in no mutations in samples belonging to 50% of the patients.

In terms of histologic grade and AJCC staging of chemoresistent tumors, 75% (6/8) of the patients had malignancies that were histological grade 2 and 25% (2/8) of the patients had malignancies that were histological grade 3. Fifty percent (4/8) of the patients had malignancies that were Stage III (3@ T3N1M0, 1 @ T2N2M0), 38% (3/8) had malignancies that were Stage II (3@ T3N0M0) and 12% (1/8) had a malignancy that was Stage IV (T3N2M1).

3.3.14 Characterization of Malignancies That Mutations Weren't Detected In

Twenty-two percent of the patients in our cohort had malignancies that we were unable to identify mutations in (Table A.17). Of these, 26% were from patients with metastatic CRC. Seventy-eight of these samples belonged to male patients, with an average age of 68 years of age. These tumors were distributed across the following anatomic sites: 44% rectum, 18% sigmoid, 11% hepatic flexure, 7% transverse, 4% ascending, 7% cecum, 4% overlapping lesion, 4% right colon. Eighty-one percent of these malignancies were histological grade 2, with a majority being AJCC stage I or II.

3.3.15 Characterization of Patients with Cancer-related Cause of Death

Thirty-seven percent (44/120) of the patients in our cohort passed away from cancer-related causes, with 48% (21/44) male and 52% (23/44) female. Thirty-four

percent of these patients (15/44) were initially diagnosed with mCRC, whereas 57% (25/44) originally were diagnosed with localized cancer or had synchronous &/or recurrent cancer (9%, 4/44). Anatomically, the tumors distributed as follows: cecum 16% (7/44), ascending 9% (4/44), hepatic flexure 7% (3/44) , transverse 14% (6/44), splenic flexure 5% (2/44), descending 7% (3/44), sigmoid 11% (5/44), rectum 18% (8/44), overlapping lesion 2% (1/44), ileocecal valve 2% (1/44), right colon 7% (3/44), and colon 2% (1/44). In terms of sidedness of the colon, 43% (19/44) of the tumors in this category arose in the proximal colon, 23% (10/44) arose in the distal colon, 14% (6/44) were located in the transverse colon and 18% (8/44) were in the rectum (Table A.18).

Of the patients who originally had metastatic disease and passed away due to cancer-related causes, 53% were female (8/15) and 46% were male (7/15), with an average age of 71 years and 67 years at the time of diagnosis, respectively. Interestingly, although these patients had mCRC, 33% had tumors that only demonstrated regional disease, with AJCC staging T3N1M0 (stage III). The tumors in this subgroup were comprised of the following mutations: BRAF V600E (20%), KRAS G12x (27%), PDL-1 expression (7%), PIK3CA G1049R (7%), PIK3CA N345K (7%), NRAS Q61x (7%), KRAS A146x (7%), KRAS K117x (7%). Interestingly, 27% (4/15) of the tumors in this subset had no mutations identified.

Of the patients who originally had localized disease, 55% were female (6/11) and 45% were male (5/11), with an average age of 75 years and 65 years at the time of diagnosis, respectively. Twenty-seven percent (3/11) of the patients in this subset went on

to develop secondary malignancies in the lung, pelvis or small intestine. The primary tumors primarily arose in the transverse, ascending colon and hepatic flexure, but tumors were also noted in the cecum, sigmoid. Forty-four percent of the tumors were AJCC stage II (T3N0M0). The tumors from this subgroup were comprised of the following mutations: 45% BRAF V600E, 27% MMR showing loss of MLH1/PMS2, 27% PDL-1 expression in the tumor cells, 18% PIK3CA E545x, and 9% had a PIK3CA H1047x or KRAS G12x mutation. Interestingly, 27% (3/11) of the tumors in this subset had no mutations identified.

The sample size (5/44) was very small for the patients who originally had refractory CRC or synchronous malignancies and died from cancer-related causes. The 13 samples for this subset were predominately grade 2 lesions with AJCC staging ranging from stage I -III. Interestingly, many of the recurrent malignancies demonstrated a similar mutation pattern compared to the original cancer.

3.4 Discussion:

Although numerous studies have focused on colorectal cancer, to our knowledge, this is the first study to characterize the molecular mutations and clinicopathological attributes of colon cancer in patients from the Upper Peninsula of Michigan. Based on our data, the Upper Peninsula has a higher incidence of primary CRC arising in the proximal colon compared to percentages published in the literature[73]. This finding doesn't appear to be biased by the composition of our cohort, as there was a higher percentage of male patients (58%) vs female patients (42%) in our study.

The prevalence of KRAS, NRAS, BRAF, and PIK3CA mutations in our cohort concurred with the frequencies published in the literature, as did the MMR / MSI status. Although not statistically significant, we observed KRAS G12x and G13x mutations in mCRC in our study, which anecdotally correlates with the poor outcomes described by other bodies of work[39,34,35]. Additionally, PIK3CA mutations, most notably the H1047x and E545x, were associated with a poor prognosis. Forty-two percent (8/19) of the patients with a PIK3CA mutation died from cancer-related causes and most had tumors arising in the transverse, descending and sigmoid colon. We also noted that more than half of the tumors with a PIK3CA mutation had a concomitant mutation. These observations are consistent with those described in the literature[74], [75], [36].

We observed a relationship between BRAF and MMR / MSI, with a higher incidence of MMR in female patients ($p= 0.02$ and $p=0.001$, respectively) with malignancies arising in the ascending colon, as also described in the literature[76]. Additionally, as Rosenbaum, et al also noted, we observed a relationship between tumors exhibiting PD-L1 expression and BRAF V600E mutation [24]. Further, our study demonstrated a concomitant KRAS and BRAF mutation which conflicts with the theory that these two mutations occur mutually exclusive of each other[71]. This phenomenon, albeit extremely rare, had been observed by other researchers[77], [78].

Although only 6% of the patients in our cohort had an NRAS mutation, the NRAS Q61x was the most frequently identified NRAS mutation and was associated with tumors arising throughout the colon. Seventy-one percent of all of the NRAS mutations in this

cohort were associated with localized disease (Stage I-II), as noted in previous works by Takane, et al and Ahmed, et al[5,35].

We had the opportunity to characterize synchronous malignancies belonging to 6 individuals in our cohort. Interesting, the male-to-female ratio was even and there was a predilection for synchronous tumors to arise in the proximal colon (i.e. ileocecal, cecum and ascending). While the remaining tumors arose in the distal (descending and sigmoid) and transverse colon, 42% and 8%, respectively, none were associated with the rectum. We observed that a majority of these tumors had intact MMR proteins and the paired synchronous samples that had identical mutations arose in the ascending and transverse colon whereas those with divert mutations arose in the descending and sigmoid colon. Although synchronous cancers may be found throughout the colon, our findings correlate with work performed by Lam et al, from the observation that synchronous cancers were primarily noted in the proximal colon[75]. We differed in our gender observations and the predilection for MMR, but this discrepancy is likely due to our small sample size.

We also had a subset of patients that experienced refractory malignancies. This portion of the cohort was too small to make anatomic site comparisons, but we did notice mutation patterns in the matched samples. Specifically, we noticed that the synchronous malignancies had identical or similar mutation patterns in the matched samples. This observation was also noted in patients who experienced recurrent CRC after having synchronous malignancies. We also noticed that several of the samples had deficient MMR, but this isn't generalizable due to our small sample size.

From a clinicopathological perspective, the chemoresistent patients (7%) in our cohort were as challenging to characterize as they likely were to treat. We observed a RAS family mutation in half of these patients, with no mutations identified in the remaining 50%. Seventy-five percent had tumors that were histological grade 2 and 50% had regional lymphatic invasion. Half of this sample set received surgical, chemotherapeutic and radiotherapy and the other 50% received surgical, chemotherapeutic therapies followed by observation. Similarly, we observed a subset of malignancies that we were unable to identify mutations in. Interestingly, a majority of these tumors belonged to males, with 44% of these tumors originating in the rectum. A majority of these tumors were grade 2 and represented localized disease. Together, we believe these findings correlate with the heterogeneity of CRC and the treatment challenges it poses[23], [79].

3.5 Limitations of the Study

Although there were many strengths of this study, there were also numerous limitations as well. We had a relatively small sample size and didn't have access to a detailed clinical history for the patients in our cohort. Therefore, we don't know if the patients in our study had a history of diabetes, inflammatory bowel disease or previous cancer. We also don't know our cohort's genetic background, ethnicity, dietary habits, and exposure to tobacco, alcohol, or survival details from diagnosis to death. We didn't have access to information regarding the ischemia time post specimen collection or about the storage conditions of our tissue blocks. Because we wanted to correlate the frequency

and diversity of mutations with clinicopathological data, there also may be some selection bias associated with our study.

3.6 Conclusion

In this novel population-based study, we comprehensively analyzed mutations in the MAPK, PIK3CA, and DNA mismatch repair pathways and correlated our findings with the clinicopathological attributes belonging to the patients in our cohort. From a primary tumor location perspective, we noted that there is a predilection for primary CRC to arise in the proximal colon of patients in the Upper Peninsula of Michigan. From a mutation status and patient outcomes perspective, although anecdotal due to the small size of our cohort, we observed PIK3CA (H1047x, E545x) and KRAS (G12x, G13x) mutations to be associated with a poor prognosis, including cancer-related death.

We also had the opportunity to analyze mutations at various stages of disease in matched samples, including biopsies, resections, metastatic disease and synchronous and recurrent malignancies. We largely observed an analogous mutational status among matched patient samples which may be clinically informative regarding treatment strategies for refractory malignancies. In total, we believe our work will inspire future patient and clinician educational initiatives and research endeavors. Additionally, this work may facilitate the development of future companion diagnostic tests and improved patient management strategies.

3.7 Future Opportunities

This study may provide the catalyst for several future studies. The increased number of CRC arising in younger patients, the predominance of primary tumors arising in the proximal colon and the observation that specific KRAS and PIK3CA mutations may be associated with an inferior patient prognosis provide justification and will hopefully spark additional prospective research endeavors. Additionally, the subset of malignancies with mutations that precluded detection in our study present a unique challenge and research opportunity for future work.

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A Figures and Tables

A.1 Incidence of Colorectal Cancer in the Upper Peninsula of Michigan.

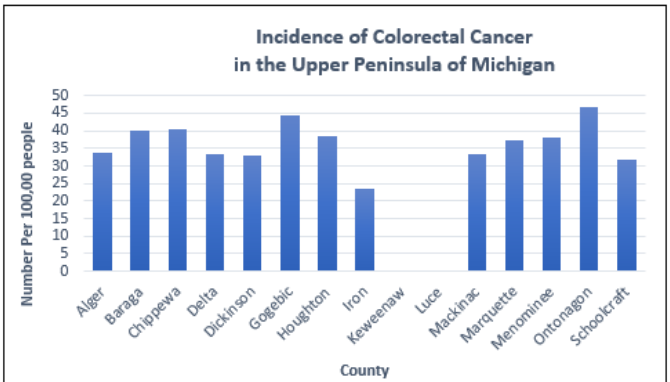

Figure A.1. Incidence of Colorectal Cancer By County in the Upper Peninsula of Michigan. This chart depicts the number of residents diagnosed with colorectal cancer by county during the 2012-2016 surveillance period. Counties with fewer than 20 cases were not included in the data set. Source: Michigan Cancer Atlas & Michigan Cancer Surveillance Program.

A.2 Percent of Upper Michigan Residents by County Diagnosed with Advanced Disease.

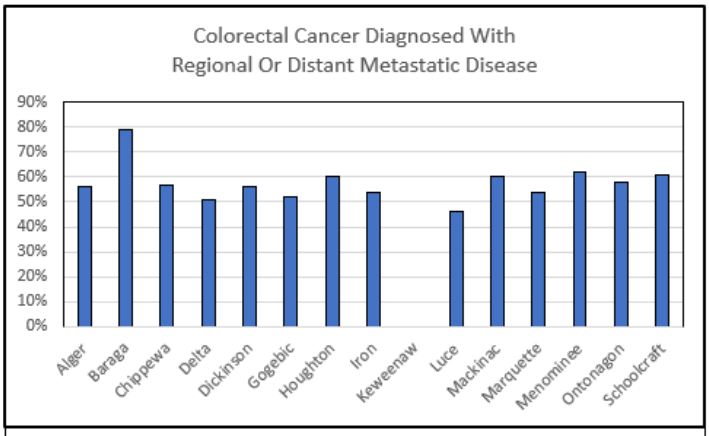

Figure A.2. Percent of Upper Michigan Residents By County Diagnosed With Advanced Disease. This chart depicts the percentage of residents diagnosed with regional or distant metastatic colorectal cancer by county during the 2012-2016 surveillance period. Counties having fewer than 20 cases during the evaluation period were excluded from the data set. Source: Michigan Cancer Atlas and the Michigan Cancer Surveillance Program.

A.3 Patient Demographics & Anatomic Distribution of Primary CRC Malignancies.

Table A.1. Patient Demographics and Anatomic Distribution of Primary Colorectal Malignancies.

Primary Site of Malignancy	Number of Subjects (n=541)	% of Total Cohort	# Males	% Males	Average Age	# Females	% Females	Average Age
Cecum	74	14%	31	42%	69	43	58%	73
Ascending Colon	68	12%	29	43%	70	39	57%	74
Hepatic flexure	11	2%	9	82%	71	2	18%	65
Transverse Colon	58	11%	35	60%	69	23	40%	72
Splenic Flexure	13	2%	7	54%	65	6	46%	67
Descending Colon	14	3%	4	29%	69	10	71%	74
Sigmoid Colon	99	18%	65	66%	62	34	34%	64
Rectum	121	22%	77	64%	67	44	36%	67
Colon, NOS	34	22%	21	62%	66	13	38%	65
Left colon	4	1%	2	50%	64	2	50%	61
Right colon	28	5%	14	50%	68	14	50%	76
Ileocecal valve	6	1%	3	50%	73	3	50%	74
Appendix	7	1%	5	71%	60	2	29%	40
Overlapping Lesion	3	.05%	1	33%	56	2	67%	77
Anal-rectal junction	1	0.1%	0	0		1	100%	51

A.4 Anatomic Distribution of Primary Colorectal Cancer in Patient Cohort.

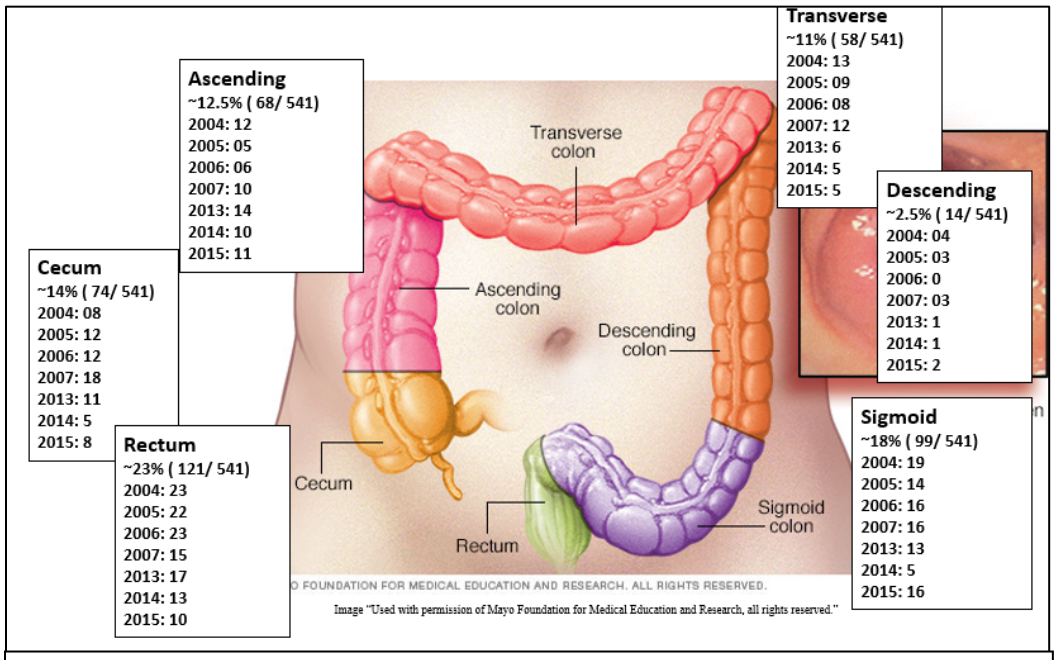

Figure A.3. Anatomic Distribution of Primary Colorectal Cancer in Patient Cohort.

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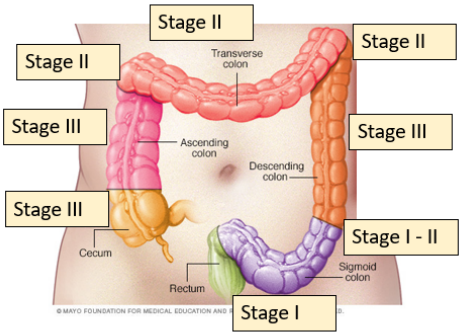
A.5 AJCC (7th ed) Staging by Anatomic Site of Primary Malignancy.

AJCC Staging By Anatomic Site of Primary Malignancy:

Region of Colon Affected / Primary Site	Summary of AJCC Staging By Primary Site
Cecum (74)	(15/74; 20%) Stage I (17/74; 23%) Stage II (27/74; 36%) Stage III (11/74; 15%) Stage IV (3/74; 11%) Stage X (1/74; 1%) Lymphoma
Ascending (68)	(1/68; 1%) Stage 0 (17/68; 25%) Stage I (18/68; 26%) Stage II (20/68; 29%) Stage III (5/68; 7%) Stage IV (7/68; 10%) Stage X
Transverse (58)	(5/58; 9%) Stage I (22/58; 38%) Stage II (17/58; 29%) Stage III (9/58; 15%) Stage IV (5/58; 9%) Stage X
Descending (14)	(1/14; 7%) Stage 0 (4/14; 29%) Stage I (2/14; 14%) Stage II (5/14; 36%) Stage III (1/14; 7%) Stage IV (1/14; 7%) Stage X
Sigmoid (99)	(27/99; 27%) Stage I (27/99; 27%) Stage II (26/99; 26%) Stage III (11/99; 11%) Stage IV (8/99; 8%) Stage X
Rectum (123)	(1/123; 1%) Stage 0 (29/123; 24%) Stage I (22/123; 18%) Stage II (22/123; 18%) Stage III (6/123; 5%) Stage IV (42/123; 34%) Stage X (1/123; 1%) Squamous cell ca
Appendix (7)	(2/7; 28%) Stage I (1/7; 14%) Stage II (2/7; 28%) Stage IV (2/7; 28%) Stage X



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Region of Colon Affected / Primary Site	Summary of AJCC Staging By Primary Site
Colon, NOS (34)	(1/34; 3%) Stage 0 (3/34; 9%) Stage I (7/34; 21%) Stage II (9/34; 26%) Stage III (6/34; 18%) Stage IV (7/34; 21%) Stage X (1/34; 3%) Burkitt's lymphoma
Right Colon (28)	(4/28; 14%) Stage I (11/28; 39%) Stage II (7/28; 25%) Stage III (6/28; 21%) Stage IV
Splenic Flexure (12)	(1/12; 8%) Stage I (7/12; 58%) Stage II (1/12; 8%) Stage III (2/12; 17%) Stage IV (1/12; 8%) Stage X
Hepatic Flexure (10)	(1/10; 10%) Stage I (4/10; 40%) Stage II (3/10; 30%) Stage III (1/10; 10%) Stage IV (1/10; 10%) Stage X
Left Colon (4)	(1/4; 25%) Stage II (3/4; 75%) Stage IV
Ileocecal valve (7)	(1/7; 14%) Stage I (1/7; 14%) Stage II (4/7; 57%) Stage III (1/7; 14%) Stage X
Overlapping Lesion, NOS (3)	(1/3; 33%) Stage II (2/3; 66%) Stage III

Figure A.4. AJCC Staging By Anatomic Site of Primary Malignancy in Patient Cohort. A majority of the malignancies arising in the proximal colon represented regional disease, with more localized disease noted from the hepatic flexure to the splenic flexure. Additionally, a majority of the malignancies in the distal colon and rectum represented localized disease, with the exception being the descending colon.

A.6 Classification of Colonoscopies and Mechanism for Detection of CRC for Patients In This Cohort.

Table A.2. Classification of Colonoscopies and Mechanism for Detection of CRC For Patients in this Cohort.
Gastroenterologist procedural notes and cancer registry data classified the colonoscopies performed on patients in this study and provided insight regarding the mode of diagnosis for the CRC samples analyzed in this study.

Colonoscopy, NOS	88	16%
Screening Colonoscopy	13	2%
Screening Colonoscopy with Biopsy	7	1%
Diagnostic Colonoscopy	132	24%
Diagnostic Colonoscopy with Biopsy	130	24%
EGD and Colonoscopy	14	2%
EGD and Colonoscopy with Biopsies	25	5%
Sigmoidoscopy	5	1%
Abnormal CT of Abdomen	1	0.2%
Bilateral Pulmonary Nodules and Large Bowel Obstruction	1	0.2%
Abnormal Virtual Colonoscopy	1	0.2%
CT-guided Biopsy	1	0.2%
Colon and Liver Biopsy	1	0.2%
Exploratory Laparotomy	7	1%
Appendix, omentum, peritoneum biopsy	1	0.2%
Patient unable to tolerate prep	3	0.5%
Colonoscopy unsuccessful, NOS	1	0.2%
Unknown / None	110	20%

A.7 Distribution of Anatomic Site of Primary Malignancy in Patient Cohort.

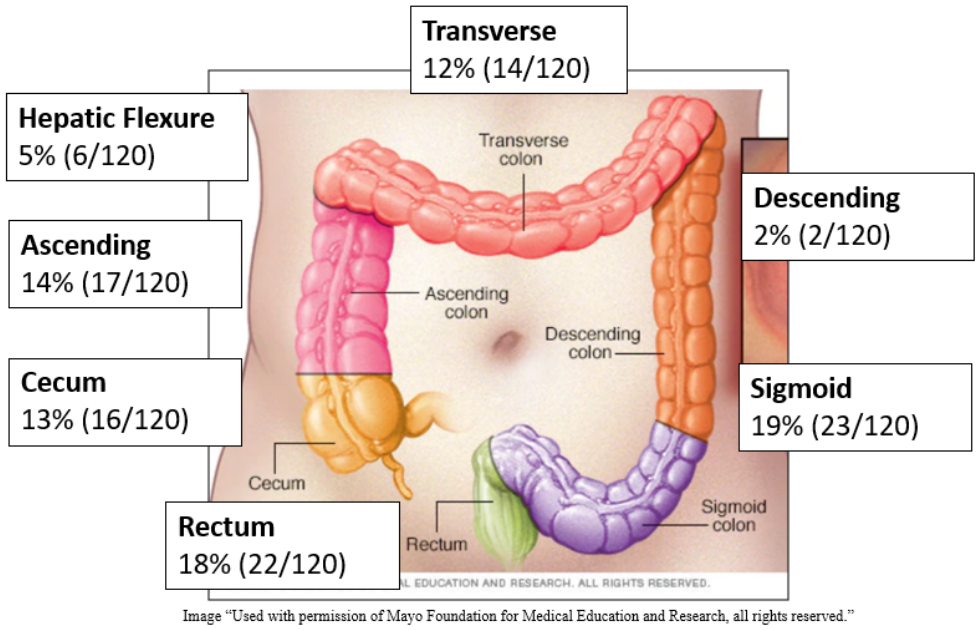

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Figure A.5. Distribution of Anatomic Site of Primary Malignancy in Patient Cohort. The primary malignancies belonging to patients in this cohort were distributed across the anatomic sites in the colon.

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A.8 Distribution of Primary Malignancies in the Proximal vs. Distal Colon for Patient Cohort.

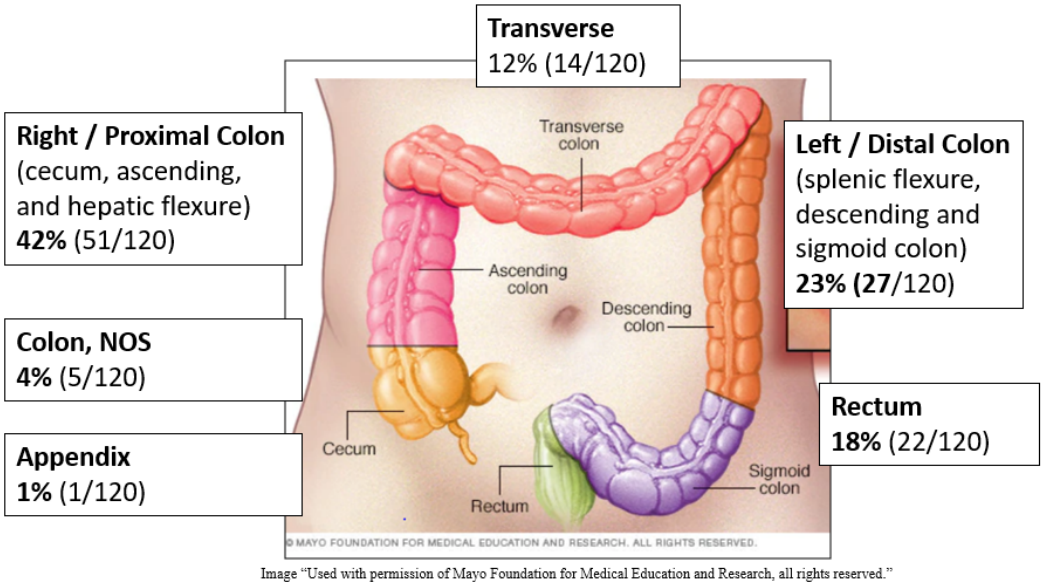

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Figure A.6. Distribution of Primary Malignancies in the Proximal vs. Distal Colon for Patient Cohort. A majority of the malignancies belonging to patients in this cohort arose in the proximal colon. While patients and matched samples were randomly selected for mutation studies, the distribution of primary malignancies is representative of the larger cohort's retrospective review.

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A.9 AJCC Staging by Primary Site for 120 Patient Cohort.

Table A.3. AJCC Staging by Primary Site for 120 Patient Cohort.
For this subset of patients, a majority of the malignancies arising in the the cecum, sigmoid colon and rectum represented regional disease. Conversely, those arising in the ascending and transverse colon represented localized disease.

Region of Colon Affected / Primary Site	Summary of AJCC Staging By Primary Site
Cecum (16)	Stage I: 25% (4/16) Stage II: 25% (4/16) Stage III: 44% (7/16) Stage IV: 6% (1/16)
Ascending (17)	Stage I: 41% (7/17) Stage II: 29% (5/17) Stage III: 24%% (4/17) Stage IV: 6% (1/17)
Transverse (14)	Stage I: 14% (2/14) Stage II: 50% (7/14) Stage III: 29% (4/14) Stage IV: 7% (1/14)
Descending (2)	Stage I: 50% (1/2) Stage III: 50% (1/2)
Sigmoid (23)	Stage I: 48% (11/23) Stage II: 17% (4/23) Stage III: 30% (7/23) Stage IV: 4% (1/23)
Rectum (22)	Stage I: 18% (4/22) Stage II: 32% (7/22) Stage III: 45% (10/22) Stage IV: 5% (1/22)
Appendix (1)	Stage I= 100% (1/1)
Colon, NOS (5)	Stage I: 20% (1/5) Stage II: 80% (4/5)
Right Colon (9)	Stage I: 33% (3/9) Stage II: 67% (6/9)

A.10 Correlation of KRAS G12x Targeted Region Mutations with Clinicopathological Data for the Patient Cohort.

Table A.4. Correlation of KRAS G12x Targeted Region Mutations with Clinicopathological Data for the Patient Cohort.
KRAS G12x was the most common KRAS mutation identified in our cohort. From a clinico-pathological perspective, this mutation was observed in a higher frequency in primary tumors arising in the proximal colon. The KRAS G12x mutation was primarily identified in Grade 2, Stage II adenocarcinoma.

KRAS Mutation	Patient Demographics			WHO Grade	AJCC Staging	Cause of Death
	Source	Gender	Age			
G12x						
	Cecum	Male	70	Grade 2	T3N2M1 Stage IV	Cancer-related
	Rectum	Male	35	Grade 2	T3N1M0 Stage III	Unknown
	Ascending	Male	50	Grade 2	T2N1M0 Stage III	Not cancer-related
	Transverse	Female	66	Grade 2	T3N1M0 Stage III	Cancer-related
	Sigmoid	Female	74	Grade 2	T4N1Mx Stage III	Cancer-related
	Transverse	Female	66	Grade 2	T3N1M1 Stage IV	Cancer-related
	Ascending	Female	90	Grade 3	T3N2M0 Stage III	Unknown
	Ascending	Female	48	Grade 2	T3N0M0 Stage II	Not applicable
	Rectum	Female	83	Grade 2	T3N0M0 Stage II	Not applicable
	Colon	Female	73	Grade 2	T1N0M0 Stage I	Unknown
	Sigmoid	Male	68	Grade 3	T1N0M0 Stage I	Cancer-related
	Ascending	Female	83	Grade 2	T2N0M0 Stage I	Not cancer-related
	Ascending	Male	67	Grade 2	T2N0M0 Stage I	Unknown
	Transverse	Female	69	Grade 2	T3N0M0 Stage II	Not cancer-related
	Transverse	Female	76	Grade 4	T3N0M0 Stage II	Cancer-related
	Colon, NOS	Male	75	Grade 2	T3N0M0 Stage II	Not cancer-related
	Sigmoid	Male	64	Grade 2	T3N0M0 Stage II	Unknown
	Cecum	Female	55	Grade 2	T2N0M0 Stage I	Not applicable

A.11 Correlation of KRAS G12x Targeted Region Mutations (continued)

Table A.4. Correlation of KRAS G12x Targeted Region Mutations with Clinicopathological Data for the Patient Cohort (continued)

KRAS Mutation	Patient Demographics			WHO Grade	AJCC Staging	Cause of Death
	Source	Gender	Age			
G12x						
	Rectum	Male	72	Grade 2	T1N0M0 Stage I	unknown
	Cecum	Male	62	Grade 3	T3N0M0 Stage II	Unknown
	Cecum	Male	57	Grade 3	T3N0M0 Stage II	Unknown
	Cecum	Male	88	Grade 2	T2N0M0 Stage I	Cancer-related
	Splenic flexure	Male	89	Grade 2	T4N0M0 Stage II	Cancer-related
	Appendix	Male	60	Grade 2	T1N0M0 Stage I	Not cancer-related
	2004-rectum	Female	90	Grade 2	T3NxMx Stage II	Cancer-related
	2005-Rectum			Grade 2	T3NxMx Stage II	See above-recurrent cancer
	Left colon, NOS	Male	78	Grade 2	T4N2M0 Stage III	Cancer-related
	Sigmoid	Female	74	Grade 3	T2N0Mx Stage I	Unknown

A.12 Correlation of KRAS G13x Targeted Region Mutations with Clinicopathological Data for the Patient Cohort.

Table A.5. Correlation of KRAS G13x Targeted Region Mutations with Clinicopathological Data for the Patient Cohort
KRAS G13x was the second most frequently identified KRAS mutation in our cohort. From a clinico-pathological perspective, this mutation was observed in a higher frequency in primary tumors arising in the distal colon. The KRAS G13x mutation was primarilyidentified in Grade 2, Stage III adenocarcinoma.

KRAS Mutation	Patient Demographics			WHO Histological Grade	AJCC Staging	Cause of Death
	Source	Gender	Age			
G13x	Hepatic flexure	Female	63	Grade 2	T4N1M0 Stage III	Not applicable
	Sigmoid	Female	45	Grade 2	T3N1M0 Stage III	Unknown
	Cecum	Female	77	Grade 2	T2N1M0 Stage III	Not cancer-related
	Transverse	Male	51	Grade 2	T2N0M0 Stage I	Not applicable
	Splenic flexure	Male	65	Grade 2	T2N0M0 Stage I	Not applicable
	Sigmoid	Male	83	Grade 2	T2N0M0 Stage I	Unknown
	Hepatic flexure	Male	84	Grade 2	T2N0M0 Stage I	Cancer-related
	Splenic flexure	Female	67	Grade 2	T4N0M0 Stage II	Cancer-related
	05-Sigmoid	Female	70	Grade 2	T3N2Mx Stage III	Cancer-related
	06-Rectum			Grade 2	T3N2Mx Stage III	Same patient - recurrent cancer
	07-Rectum			Grade 2	T3N2Mx Stage III	Same patient - recurrent cancer

A.13 Correlation of KRAS G13x and Less Common Targeted Region Mutation with Clinicopathological Data for the Patient Cohort (continued).

Table A.5. Correlation of KRAS G13x Targeted Region Mutations with Clinicopathological Data for the Patient Cohort (continued)

KRAS Mutation	Patient Demographics			WHO Histological Grade	AJCC Staging	Cause of Death
	Source	Gender	Age			
G12x & G13x	Ascending	Female	86	Grade 2	T1N0M0 Stage I	Cancer-related
A146x	Ascending	Male	53	Grade 2	T3N2M1 Stage IV	Cancer-related
	Transverse	Male	72	Grade 2	T4N0M0 Stage II	Not cancer-related
K117x	Rectum	Male	68	Grade 2	T3N2M1 Stage IV	Cancer-related COD
	Sigmoid	Male	59	Grade 3	T3N1M0 Stage III	Unknown
Q61x	Ascending	Male	67	Grade 2	T3N0M0 Stage II	Not cancer-related
A59x	Sigmoid	Male	48	Grade 3	T2N0M0 Stage I	Unknown

A.14 Correlation of BRAF V600E Mutation with Clinicopathological Data for the Patient Cohort.

Table A.6. Correlation of BRAF V600E Mutation with Clinicopathological Data for the Patient Cohort
From a clinico-pathological perspective, the BRAF V600E mutation was observed in a higher frequency in female patients (p=0.001) and in primary tumors arising in the proximal colon. The BRAF V600E mutation was primarily identified in Grade 2 or 3 adenocarcinoma with localized disease.

Patient Demographics			**WHO**		
Source	**Gender**	**Age**	**Histological Grade**	**AJCC Staging**	**Cause of Death**
Cecum	Female	81	Grade 3	T4N2M0 Stage III	Not cancer-related
Cecum	Female	71	Grade 3	T3N1M0 Stage III	Cancer-related
Cecum	Female	66	Grade 2	T3N1M0 Stage III	Cancer-related
Descending	Female	60	Grade 4	T4N1M0 Stage III	Cancer-related
Ascending	Female	90	Grade 3	T3N2M0 Stage III	unknown
Ascending	Male	69	Grade 2	T2N0M0 Stage I	Not applicable
Transverse	Female	48	Grade 2	T3N0M0 Stage II	Unknown
Sigmoid	Female	82	Grade 2	T1N0M0 Stage I	Cancer-related
Right colon	Male	83	Grade 2	T3N0Mx Stage II	Cancer-related
Splenic flexure	Male	64	Grade 1	T3N0M0 Stage II	Unknown
Ascending	Female	87	Grade 2	T3N0M0 Stage II	Cancer-related
Descending	Female	69	Grade 2	T2N0M0 Stage I	Cancer-related
Transverse	Male	64	Grade 2	T3N0M0 Stage II	Cancer related
Cecum	Male	66	Adeno, NOS	T3N0M0 Stage II	unknown
Ascending	Female	78	Grade 4	T3N0M0 Stage II	Cancer-related
Transverse	Female	75	Grade 2	T3N0M0 Stage II	Cancer-related

A.15 Correlation of BRAF V600E Mutation with Clinicopathological Data for Patient Cohort (continued).

Table A.6. Correlation of BRAF V600E Mutation with Clinicopathological Data for the Patient Cohort (continued)

Patient Demographics			**WHO Histological Grade**	**AJCC Staging**	**Cause of Death**
Source	**Gender**	**Age**			
Transverse	Female	83	Grade 3	T4N0M0 Stage II	Not cancer-related
Cecum	Female	83	Grade 3	T3N0M0 Stage II	Cancer-related
Splenic flexure	Female	87	Grade 2	T3N0Mx Stage II	Not cancer-related
Right colon	Female	80	synch #2- Grade 2 #6- Grade 1 Recurrent resection & biopsy Grade 3	T3N0Mx Stage II T1N0Mx Stage I T3N0Mx Stage II	Cancer-related
Synchronous Ascending (block #3) Transverse (block #7)	Female	70	Sync #3-Grade 3 #7-Grade 2	T2N0M0 Stage I	Not applicable

A.16 Correlation of NRAS Targeted Region Mutations with Clinicopathological Data for the Patient Cohort.

Table A.7. Correlation of NRAS Targeted Region Mutations with Clinicopathological Data for the Patient Cohort
NRAS mutations in codon 61were the most frequently NRAS mutations identified in our cohort. NRAS mutations were observed in a higher frequency in male patients and were slightly more prevalent in primary tumors arising in the proximal colon. NRAS mutations primarily identified in Grade 2 adenocarcinoma with localized disease.

NRAS Mutation	**Patient Demographics**			**WHO Histological Grade**	**AJCC Staging**	**Cause of Death**
	Source	Gender	Age			
G13X	Rectum	Male	51	Grade 2	T3N1M0 Stage III	Unknown
Q61X	Ileocecal	Male	81	Grade 2	T2N2M0 Stage III	Cancer-related
	Right colon	Female	70	Grade 2	T3N0M0 Stage II	Unknown
	Sigmoid	Male	54	Grade 2	T2N0M0 Stage II	Not applicable
	Transverse	Female	84	Grade 3	T1N0M0 Stage I	Not cancer-related
	Colon, NOS	Male	53	Grade 2	T3N0M0 Stage II	Not applicable
G12X	Cecum	Male	76	Grade 2	T1N0M0 Stage I	Cancer-related

A.17 Correlation of PIK3CA Targeted Region Mutations with Clinicopathological Data for the Patient Cohort.

Table A.8. Correlation of PIK3CA Targeted Region Mutations with Clinicopathological Data for the Patient Cohort
PIK3CA mutations in exons 9 and 20 were the most frequently PIK3CA mutations identified in our cohort. PIK3CA mutations were observed in equally among both genders and were identified in higher frequencies in primary tumors arising in the proximal colon. PIK3CA mutations associated with Grade 2, AJCC Stage II adenocarcinoma in our cohort.

PIK3CA Mutation	Patient Demographic Source	Gender	Age	WHO Grade	AJCC Staging	Cause of Death
H1047X						
	Sigmoid	Female	82	Grade 2	T1N0M0 Stage I	Cancer-related
	Hepatic Flexure	Male	76	Grade 2	T3N0M0 Stage II	Not cancer-related
	Transverse	Male	72	Grade 2	T4N0M0 Stage II	Not cancer-related
	Transverse	Female	76	Grade 4	T3N0M0 Stage II	Cancer-related
	Cecum	Male	62	Grade 3	T3N0M0 Stage II	Unknown
	Colon	Female	67	Grade 2	T3N0M0 Stage II	Cancer-related
	Right Colon	Female	80	Grade 2	T3N0Mx Stage II	Unknown
E545X						
	Ascending	Male	53	Grade 2	T3N2M1 Stage IV	Cancer-related
	Ascending	Female	48	Grade 2	T3N0M0 Stage II	Not applicable
	Ileocecal	Male	55	Grade 2	T3N1M0 Stage III	Unknown
	Transverse	Male	64	Grade 2	T3N0M0 Stage II	Cancer-related
	Transverse	Female	76	Grade 4	T3N0M0 Stage II	Cancer-related
	Descending	Male	78	Grade 2	T4N2M0 Stage III	Cancer-related
	Ascending	Female	79	Grade 4	T3N0Mx Stage II	Not cancer-related
	Right Colon	Female	80	Grade 2	T3N0Mx Stage II	Cancer-related
G1049R	Sigmoid	Male	69	Grade 2	N/A Bx	Cancer-related

A.18 Correlation of PIK3CA Targeted Region Mutations with Clinicopathological Data for the Patient Cohort (continued).

Table A.8. Correlation of PIK3CA Targeted Region Mutations with Clinicopathological Data for the Patient Cohort (continued)

PIK3CA Mutation	Patient Demographics			WHO Histological Grade	AJCC Staging	Cause of Death
	Source	Gender	Age			
C420R	Cecum	Female	81	Grade 3	T4N2M0 Stage III	Not cancer-related
	Ascending	Male	67	Grade 2	T3N0M0 Stage II	Not cancer-related
Q546X	Cecum	Male	62	Grade 4	T1No Mx Stage I	Not applicable
	Ascending	Male	67	Grade 2	T2N0M0 Stage I	Unknown
N345K	Descending	Female	60	Grade 4	T4N1M0 Stage III	Cancer-related

A.19 Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort.

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort
Twenty-nine percent of the patients in our cohort had malignancies in which multiple mutations were identified. Malignancies arising in the ascending colon demonstrated the greatest mutational diversity and the frequency of concomitant mutations gradually decreased from the transverse colon to the rectum.

Patient Demographics			**WHO Histologic Grade**	**AJCC Staging**	**Cause of Death**	**Characterization of Mutations:**
Gender	Age	Source				
Female	81	Cecum	Grade 3	T4N2M0 Stage III	Not cancer-related	MMR Status: deficient, loss of MLH1/PMS2 (bx, resection + nodes) PDL-1 expression: 30% (bx & nodes), 75% exp in resection. BRAF V600E/E2/D Positive via IHC and PCR (bx, resection, nodes) PIK3CA C420R (bx, resection + nodes).
Male	53	Ascending	Grade 2	T3N2M1 Stage IV	Cancer-related	Resection & nodes: Positive for KRAS A146x mutation Nodes: positive for PIK3CA E545x mutation.

A.20 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Female	60	Descending	Grade 4	T4N1M0	Cancer-related	Bx: BRAF V600E positive via IHC only; no mut detected via PCR. No PIK3CA mutation detected. Resection: BRAF V600E via IHC and PCR. PIK3CA mutation (N345K) detected. Nodes: BRAF V600E via IHC only; no mut detected via PCR.
Female	90	Ascending	Grade 3	T4N1M0	Unknown	Bx: MMR Status: Deficient / loss of MLH1/PMS2 BRAF V600E positive via IHC only; PCR was invalid. PDL-1 expression= <1% Resection: BRAF V600E positive via IHC and PCR. KRAS mutation (G12x) detected. Nodes: BRAF V600E + via IHC and PCR.

A.21 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Female	48	Ascending	Grade 2	T3N0M0	Not applicable	Bx: KRAS mutation invalid PIK3CA- no mutation detected Resection: KRAS mutation (G12x) detected. PIK3CA mutation (E545x) detected.

A.22 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Male	83	Right Colon	Grade 2	T3N0Mx Stage II	Cancer-related	Bx: MMR Status: Deficient /loss MLH1/PMS2 PDL-1 expression: <1% BRAF V600E positive via IHC and PCR. Resection: MMR Status: Deficient / loss MLH1/PMS2 PDL-1 expression: 5% BRAF V600E positive via IHC and PCR.
Male	72	Transverse	Grade 2	T4N0M0 Stage II	Not cancer-related	Bx: MMR status: Deficient / loss MLH1/PMS2 Resection: MMR status: Deficient / loss MLH1/PMS2 KRAS mutation (A146x) detected PIK3CA mutation (H1047x) detected

A.23 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Male	64	Splenic Flexure	Grade 1 arising in tubular adenoma	T3N0M0	Unknown	Resection: PDL-1 expression in TC: 3% expression BRAF V600E positive via IHC and PCR.
Male	68	Sigmoid	Grade 3	T3N0M0	Cancer-related	Resection: PDL-1 expression in TC: 5% expression KRAS mutation (G12x) detected
Female	87	Ascending	Grade 2	T3N0M0	Cancer-related	Resection: MMR Status: Deficient / loss MLH1/PMS2 BRAF V600E positive via IHC and PCR.
Female	83	Ascending	Grade 2	T2N0M)	Not cancer-related	Resection: PDL-1 expression inTC: <1% expression KRAS mutation (G12x) detected
Female	86	Ascending	Grade 2	T1N0M0	Cancer-related	Resection: KRAS co-mutations (G12x and G13x)
Male	67	Ascending	Grade 2	T2N0M0	Unknown	Resection: PDL-1 expression in TC: <1% expression KRAS mutation (G12x) detected PIK3CA mutation (Q546x) detected

A.24 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Gender	Age	Source				
Female	69	Descending	Grade 2	T2N0M0	Cancer-related	Resection: MMR Status: Deficient / loss MLH1/PMS2 PDL-1 expression in TC: <1% expression BRAF V600E positive via IHC and PCR.
Female	76	Transverse	Grade 4	T3N0M0	Cancer-related	Resection: PDL-1 expression in TC: <1% expression KRAS mutation (G12x) detected PIK3CA co-mutations (H1047x & E545x) detected
Male	64	Transverse	Grade 2	T3N0M0	Cancer-related	Resection: BRAF V600E positive via IHC and PCR PIK3CA mutations (E545x) detected
Male	66	Cecum	Grade 2	T3N0M0	Unknown	Resection: MMR Status: Deficient / loss MLH1/PMS2 BRAF V600E positive via IHC and PCR.

A.25 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Gender	Age	Source				
Male	64	Sigmoid	Grade 2	T3N0M0	Unknown	Resection: MMR Status: Deficient / loss of MLH1/PMS2, MSH2/MSH6 BRAF V600E positive via IHC and PCR.
Female	51	Cecum	Grade 2	T2N0M0	Not applicable	Resection: PDL-1 expression in TC= 1% expression KRAS mutation (G12x) detected
Male	62	Right colon	Grade 2 (Arising in TA)	T3N0M0	Not applicable	Resection: MMR Status: Deficient / loss MLH1/PMS2) PDL-1 expression: <1%; lots in IC in stroma
Male	62	Cecum	Grade 3	T3N0M0	Unknown	Resection: KRAS mutation (G12x) detected PIK3CA mutation (H1047x) detected

A.26 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Female	67	Splenic flexure	Grade 2	T4N0M0	Cancer-related	Resection: MMR Status: Deficient / loss MSH2); lots in IC KRAS mutation (G13x) detected.
Male	88	Cecum	Grade 2	T2N0M0	Cancer-related	Resection: MMR Status: Deficient / loss MSH6 KRAS mutation (G12x) detected.
Female	67	Colon, NOS	Grade 2	T3N0M0	Cancer-related	Resection: MMR Status: Deficient / loss MLH1/PMS2 PIK3CA mutation (H1047x) detected
Male	60	Appendix	Grade 2	T1N0M0	Not cancer-related	Resection: PDL-1 expression in TC: 10-29% expression KRAS mutation (G12x) detected
Female	83	Transverse	Grade 3	T4N0M0	Not cancer-related	Resection: MMR Status: Deficient / loss MLH1/PMS2 PDL-1 expression in TC: <1% expression BRAF V600E positive via IHC and PCR.

A.27 Diversity of Concomitant Mutations and Clinicopathological Features (continued)

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Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics			**WHO Histologic Grade**	**AJCC Staging**	**Cause of Death**	**Characterization of Mutations:**
Gender	**Age**	**Source**				
Female	83	Cecum	Grade 3	T3N0M0	Cancer-related	Resection: MMR Status: Deficient / loss MLH1/PMS2 PDL-1 expression in TC: 1-9% expression BRAF V600E positive via IHC and PCR.
Female	87	Splenic Flexure	Grade 2	T3N0Mx	Not cancer-related	Resection: PDL-1 expression in TC: <1% expression BRAF V600E positive via IHC and PCR.

A.28 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Female	80	Right Colon	synch Block #2: grade 2 Block #6: grade 1 Recurrent Biopsy: Grade 3 Resection: Grade 3	T3N0Mx T1N0Mx Not staged T3N0Mx	Cancer-related	Block #2 MMR status: Loss MLH1/PMS2 PDL-1 expression in TC: 10-29% expression BRAF V600E positive via IHC and PCR PIK3CA co-mutations (H1047x & E545x) detected Block #6 MMR status: Loss MLH1/PMS2 PDL-1 expression in TC: 1-9% expression BRAF V600E positive via IHC and PCR Biopsy MMR status: Loss MLH1/PMS2) PDL-1 expression in TC= 1-9% expression BRAF V600E positive via IHC Resection: MMR status: Deficient / loss MLH1/PMS2 PDL-1 expression in TC: 10-29% expression BRAF V600E positive via IHC and PCR.

A.29 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Staging	Cause of Death	Characterization of Mutations:
Male	53	Colon, NOS	Grade 2	T3N0M0	Not applicable	Resection: MMR status: Deficient / loss PMS2 NRAS mutation (Q61x) detected
Female	70	Sigmoid and Rectum	All were grade 2	All T3N2Mx	Cancer-related	Resection: PDL-1 expression: 1-9% expression KRAS mutation (G13x) detected. Nodes: KRAS mutation (G13x) detected. Sacrum: KRAS mutation (G13x) detected. Rectum: KRAS mutation (G13x) detected
Female	70	Synchronous cancers Ascending & Transverse	Block #3: Ascending Grade 3 Block #7: Transverse Grade 2	T3N0Mx T3N0M0	Not applicable	Both blocks: MMR Status: Deficient / loss MLH1/PMS2 BRAF V600E positive via IHC

A.30 Diversity of Concomitant Mutations and Clinicopathological Features (continued).

Table A.9. Diversity of Concomitant Mutations and Clinicopathological Features for the Patient Cohort (continued)

Patient Demographics			**WHO Histologic Grade**	**AJCC Staging**	**Cause of Death**	**Characterization of Mutations:**
Gender	**Age**	**Source**				
Female	79	Synchronous cancers Ascending	Block B6: Ascending (1cm from ileocecal valve) Grade 4 Block B10: Ascending (distal to 1^0 mass) Grade 4	T3N0Mx T3N0Mx	Not cancer-related	Block B6: MSI-High (loss MLH1/PMS2) PIK3CA mutation (E545x) detected. Block B10: MSI-low (loss MLH1) PIK3CA mutation (E545x) detected.

A.31 Mismatch Repair IHC Staining Depicting Intact MMR Proteins in Colon Resection.

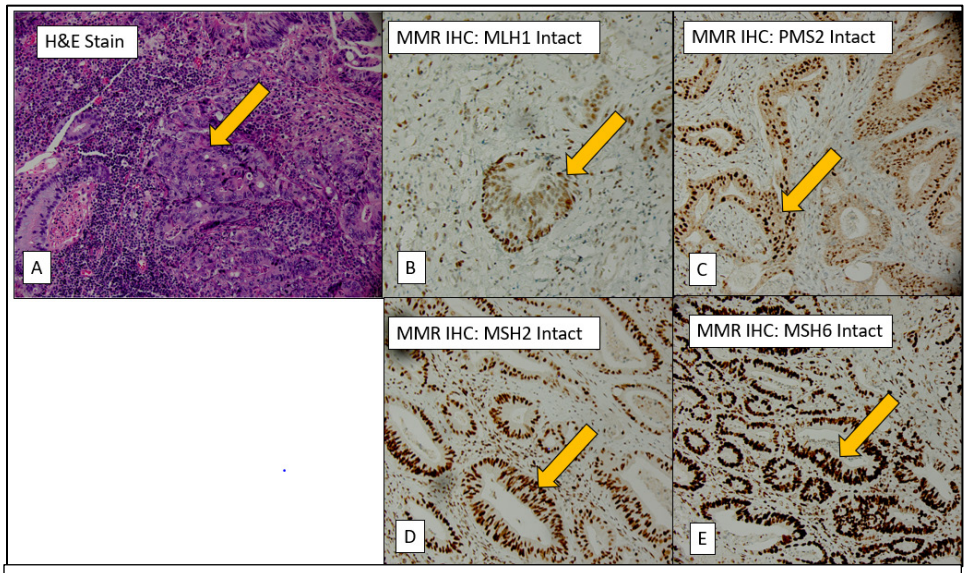

Figure A.7. Mismatch Repair IHC Staining Depicting Intact MMR Proteins in Colon Resection. **a)** Hematoxylin & Eosin stain (arrow denotes) malignant cells, **b)** MLH1 shows positive nuclear staining in tumor cells, **c)** PMS2 demonstrates positive nuclear staining in the tumor cells, **d)** MSH2 demonstrates positive nuclear staining in the tumor cells, **e)** MSH6 demonstrates positive nuclear staining in tumor cells. All images were taken at 40x magnification.

A.32 Mismatch Repair IHC Staining Depicting Intact MMR Repair Proteins in mCRC in Lymph Node.

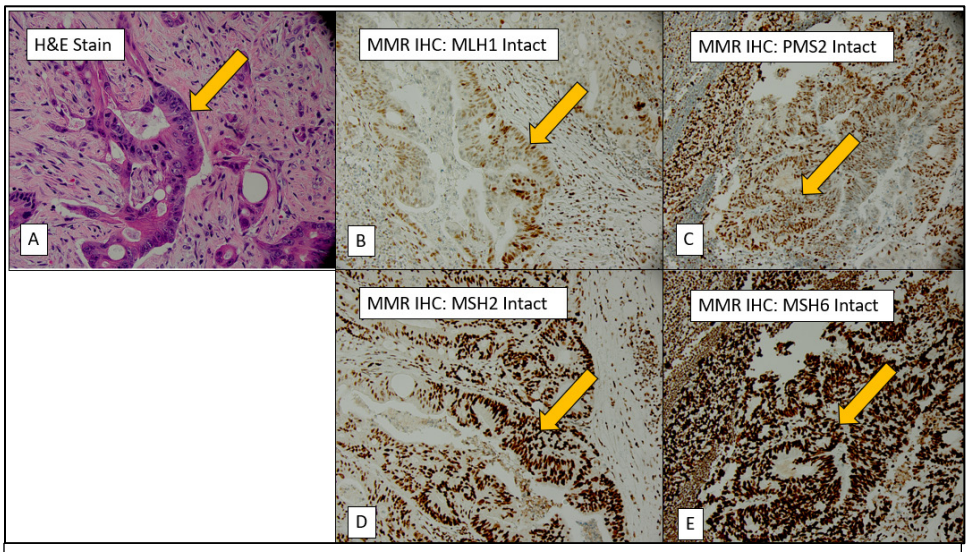

Figure A.8. Mismatch Repair IHC Staining Depicting Intact MMR Proteins in mCRC in Lymph Node. a) Hematoxylin & Eosin stain (arrow denotes) malignant cells, **b)** MLH1 shows positive nuclear staining in tumor cells, **c)** PMS2 demonstrates positive nuclear staining in the tumor cells, **d)** MSH2 demonstrates positive nuclear staining in the tumor cells, **e)** MSH6 demonstrates positive nuclear staining in tumor cells. All images were taken at 40x magnification.

A.33 Mismatch Repair IHC Staining Depicting Deficient MMR Repair Proteins in mCRC in Colon Resection.

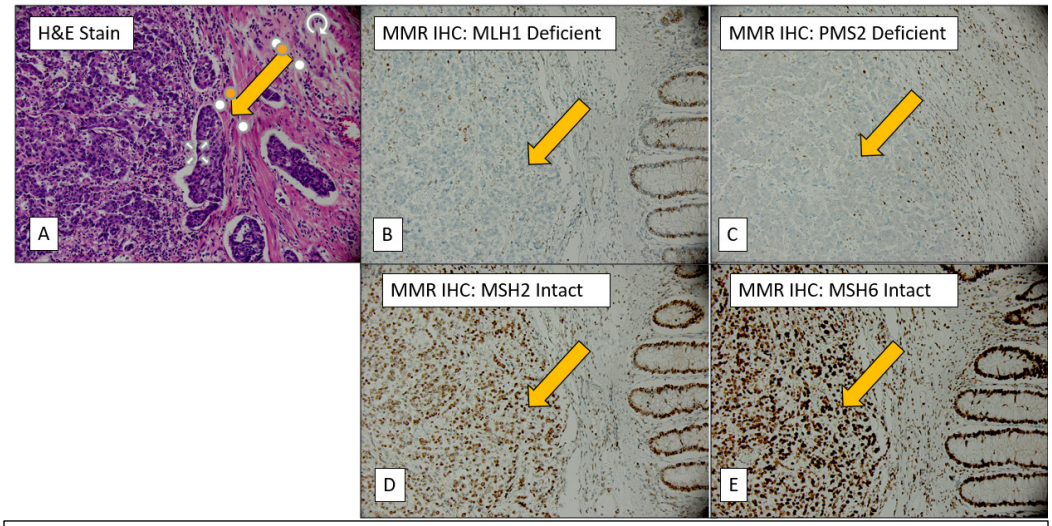

Figure A.9. Mismatch Repair IHC Staining Depicting Deficient MMR Proteins in Colon Resection. a) Hematoxylin & Eosin stain (arrow denotes) malignant cells, **b)** MLH1 shows absence of nuclear staining in tumor cells but is present in adjacent normal colonic epithelium which serves as an internal control **c)** PMS2 demonstrates absence nuclear staining in the tumor cells, **d)** MSH2 demonstrates positive nuclear staining in the tumor cells, **e)** MSH6 demonstrates positive nuclear staining in tumor cells. All images were taken at 40x magnification.

A.34 Mismatch Repair IHC Staining Depicting Deficient MMR Protein in mCRC in Lymph Node.

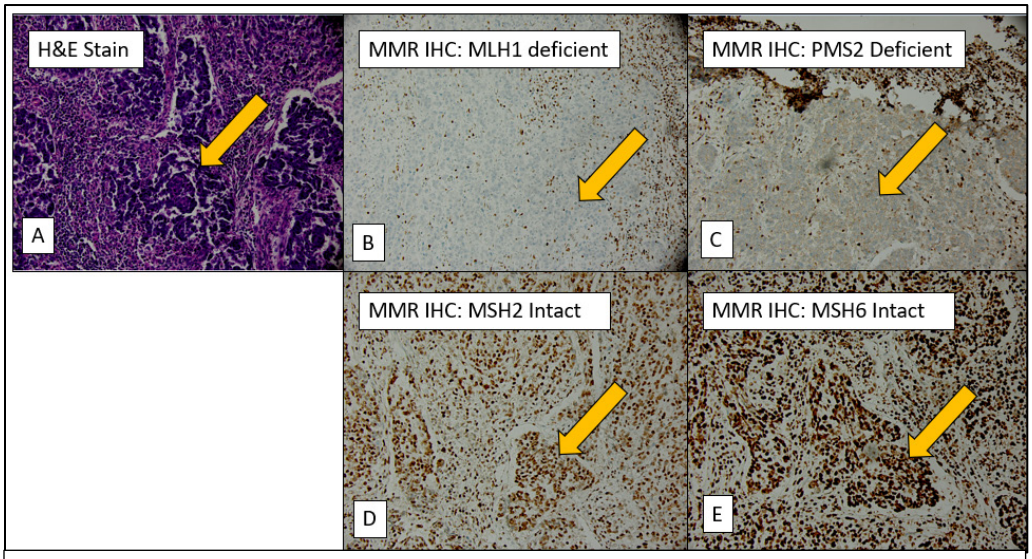

Figure A.10. Mismatch Repair IHC Staining Depicting Deficient MMR Proteins in mCRC in Lymph Node. a) Hematoxylin & Eosin stain (arrow denotes) malignant cells, **b)** MLH1 shows absence of nuclear staining in tumor cells **c)** PMS2 demonstrates absence nuclear staining in the tumor cells, **d)** MSH2 demonstrates positive nuclear staining in the tumor cells, **e)** MSH6 demonstrates positive nuclear staining in tumor cells. All images were taken at 40x magnification.

A.35 Characterization of Malignancies with Intact DNA Mismatch Repair Proteins.

Table A.10. Characterization of Malignancies with Intact DNA Mismatch Repair Proteins.

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Rectum	Male	46	Grade 3	T2 N2 M0 Stage III	Unknown
Sigmoid	Male	69	Bx: Grade 2 Resection: Grade 3	T3N1M3 Stage IV	Unknown
Cecum	Male	70	Bx: Not spec Resection: Grade 2	T3N2M1 Stage IV	Unknown
Rectum	Male	35	Grade 2	T3N1M0 Stage III	Unknown
Sigmoid	Male	61	Grade 2	T3N2M0 Stage III	Not cancer-related
Sigmoid	Male	49	Grade 2	T3N1M0 Stage III	Unknown
Hepatic flexure	Female	65	Bx: Grade 2 Resection: Grade 2	T3N1M0 Stage III	Unknown
Ascending	Male	50	Grade 2	T2N1M0 Stage III	Not cancer-related
Transverse	Female	66	Grade 2	T3N1M0 Stage III	Cancer-related
Hepatic flexure	Female	63	Grade 2	T4N1M0 Stage III	Not applicable
Rectum	Male	78	Grade 2	T2N1Mx Stage III	Unknown

A.36 Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued).

Table A.10. Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Rectum	Female	65	Grade 2	T3N1M0 Stage III	Cancer-related
Sigmoid	Female	45	Grade 2	T3N1M0 Stage III	Unknown
Cecum	Female	71	Grade 3	T3N1M0 Stage III	Cancer-related
Ascending	Male	53	Grade 2	T2N2M1 Stage IV	Cancer-related
Rectum	Male	78	Grade 3	T3N2M0 Stage III	Cancer-related
Overlapping lesion	Female	70	Grade 2	T3N1M0 Stage III	Cancer-related
Rectum	Male	68	Grade 2	T3N0M0 Stage II	Cancer-related COD
Sigmoid	Female	74	Grade 2	T4N1Mx Stage III	Unknown
Transverse	Female	66	Grade 2	T3N1M1 Stage IV	Cancer-related
Rectum	Male	51	Grade 2	T3N1M0 Stage III	Unknown
Sigmoid	Male	59	Grade 3	T3N1M0 Stage III	Unknown
Cecum	Female	66	Grade 2	T3N1M0 Stage III	Cancer-related
Descending	Female	60	Grade 4	T4N1M0 Grade III	Cancer-related
Cecum	Female	77	Grade 2	T2N1M0 Stage III	Not cancer-related
Rectum	Male	71	Grade 2	T3N1M0 Stage III	Unknown
Rectum	Male	83	Grade 3	T3N2M0 Stage III	Unknown
Ileocecal	Male	81	Grade 2	T2N2M0 Stage III	Cancer-related
Sigmoid	Male	60	Grade 2	T1N1M0 Stage III	Unknown

A.37 Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued).

Table A.10. Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Sigmoid	Male	48	Grade 2 (arising in TA)	T1N0M0 Stage I	Unknown
Ascending	Male	69	Grade 2	T2N0M0 Stage I	Not applicable
Cecum	Male	62	Bx: in-situ Grade 4	T1N0Mx Stage I	Not applicable
Right colon	Female	70	Grade 2	T3N0M0 Stage II	Unknown
Ascending	Male	67	Grade 2	T2N0M0 Stage I	Not cancer-related
Rectum	Male	68	Grade 2	T1N0M0 Stage I	Unknown
Sigmoid	Male	54	Grade 2	T2N0M0 Stage I	Not applicable
Sigmoid	Female	81	Grade 2	T3N0M0 Stage II	Not cancer-related
Rectum	Male	76	Grade 2	T3N0M0 Stage II	Not applicable
Sigmoid	Male	78	Grade 2	T3N0M0 Stage II	Cancer-related
Transverse	Female	48	Grade 2	T3N0M0 Stage II	Unknown
Sigmoid	Male	48	Grade 3	T2N0M0 Stage I	Unknown
Ascending	Female	48	Grade 2	T3N0M0 Stage II	Not applicable
Ascending	Male	67	Grade 2	T2N0M0 Stage I	Unknown
Rectum	Female	83	Grade 2	T3N0M0 Stage II	Not applicable
Transverse	Male	82	Grade 2	T4N0M0 Stage II	Cancer-related
Left colon, NOS	Female	73	Grade 2	T1N0Mx Stage I	Unknown

A.38 Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued).

Table A.10. Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued)

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Splenic flexure	Male	64	Grade 1	T3N0M0 Stage II	Unknown
Rectum	Male	55	Grade 2	T2N0M0 Stage I	Unknown
Splenic flexure	Male	65	Grade 2 (in TA)	T2N0M0 Stage I	Not applicable
Sigmoid	Male	68	Grade 3 (in TA)	T1N0M0 Stage I	Cancer-related
Rectum	Male	56	Grade 2	T3NxMx Stage II	Cancer-related
Ascending	Female	83	Grade 2	T2N0M0 Stage I	Not cancer-related
Ascending	Female	86	Grade 2	T1N0M0 Stage I	Cancer-related
Ascending	Male	67	Grade 2	T2N0M0 Stage I	Unknown
Sigmoid	Male	56	Grade 2	T2N0M0 Stage I	Not cancer-related
Transverse	Female	69	Grade 3	T3N0M0 Stage II	Deceased
Rectum	Male	33	Grade 2	T2N0M0 Stage I	Unknown
Transverse	Female	76	Grade 4	T3N0M0 Stage II	Cancer-related
Ileocecal	Male	55	Grade 3	T3N0M0 Stage II	Unknown
Rectum	Female	65	Grade 2	T3N0M0 Stage II	Cancer-related
Sigmoid	Male	83	Grade 2	T2N0M0 Stage I	Unknown
Hepatic flexure	Male	84	Grade 2	T2N0M0 Stage I	Cancer-related
Cecum	Male	76	Grade 2	T1N0M0 Stage I	Cancer-related
Colon, NOS	Male	75	Grade 2	T3N0M0 Stage II	Not cancer-related
Transverse	Male	64	Grade 3	T3N0M0 Stage II	Cancer-related
Transverse	Male	72	Grade 2	T3N0M0 Stage II	Not cancer-related

A.39 Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued).

Table A.10. Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued)

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Cecum	Female	55	Grade 2	T2N0M0 Stage I	Not applicable
Transverse	Female	84	Grade 3	T1N0M0 Stage I	Not cancer-related
Ascending	Female	66	Grade 3	T1N0M0 Stage I	Unknown
Rectum	Male	72	Grade 2	T1N0M0 Stage I	Unknown
Cecum	Male	62	Grade 3	T3N0M0 Stage II	Unknown
Sigmoid	Male	80	Grade 2	T3N0M0 Stage II	Not cancer-related
Cecum	Male	57	Grade 3	T3N0M0 Stage II	Unknown
Ascending	Female	78	Grade 4	T3N0M0 Stage II	Cancer-related
Transverse	Female	75	Grade 2	T3N0M0 Stage II	Cancer-related
Cecum	Male	83	Grade 2	T1N0M0 Stage I	Cancer-related
Hepatic flexure	Female	66	Grade 2	T3N0M0 Stage II	Cancer-related
Right colon	Male	68	Grade 2	T1N0M0 Stage I	Not cancer-related
Splenic flexure	Male	89	Grade 2	T4N0M0 Stage II	Cancer-related
Colon, NOS	Male	51	Grade 2	T3N0M0 Stage II	Cancer-related
Rectum	Male	73	Grade 2	T3N0M0 Stage II	Unknown
Hepatic flexure	Male	52	Grade 2	T3N0M0 Stage II	Unknown
Right colon	Female	73	Grade 2	T3N0M0 Stage II	Unknown
Sigmoid	Male	58	Grade 1	T1N0M0 Stage I	Not cancer-related
Appendix	Male	60	Grade 2	T1N0M0 Stage I	Not cancer-related
Splenic flexure	Female	87	Grade 2	T3N0M0 Stage II	Not cancer-related

A.40 Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued).

Table A.10. Characterization of Malignancies with Intact DNA Mismatch Repair Proteins (continued)

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Rectum	Female	90	2004 Bx: grade 2 Resection: Grade 2 2005 Grade 3	Not staged T3NxMx Stage II T3N0M0 Stage II	Cancer-related
Sigmoid and Rectum	Female	70	Sigmoid: Grade 2 Rectum: Grade 2 Rectum Grade 2	T3N2Mx T3N2Mx Stage III T3N2Mx Stage III	Cancer-related
B3- Sigmoid /ulcerating lesion B9- Sigmoid/ proximal to original bx site	Female	74	Grade 3 High-grade dysplasia	T2N0Mx Stage I	Unknown
B1-Ileocecal D1- Sigmoid Resection: Sigmoid	Male	74	Grade 2 Grade 2 Grade 2	TisNxMx Stage 0 TisNxMx Stage 0 T1N0M0 Stage I	Unknown
#3- Cecum #8- Right	Male	83	Both are Grade 2	Block #3 T2N0mx Stage I Block #8 T1N0Mx Stage I	Unknown
Descending Blocks #3 Block #8	Male	78	Grade 2	T4N2M0 Stage III	Cancer-related

A.41 Characterization of Malignancies with Deficient DNA Mismatch Repair Proteins Demonstrating a Loss of MLH1/PMS2.

Table A.11. Characterization of Malignancies with Deficient DNA Mismatch Repair Proteins Demonstrating a Loss of MLH1/PMS2

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Cecum	Female	81	Grade 3	T4N2M0 Stage III	Not cancer-related
Cecum	Female	72	Grade 2	T3N2N0 Stage III	Unknown
Cecum	Female	73	Grade 2	T4N2M0 Stage III	Unknown
Ascending	Female	90	Grade 3	T3N2M0 Stage III	Unknown
Right colon	Female	80	Grade 2	T2N0M0 Stage I	Unknown
Sigmoid	Female	82	Grade 2	T1N0M0 Stage I	Cancer-related death
Hepatic flexure	Male	76	Grade 2	T3N0M0 Stage II	Not cancer-related
Right colon	Male	83	Grade 2	T3N0Mx Stage II	Cancer-related
Transverse	Male	51	Grade 2	T2N0mx Stage I	Not applicable
Transverse	Male	72	Grade 2	T4N0M0 Stage II	Not cancer-related
Hepatic flexure	Male	56	Grade 2	T3N0M0 Stage II	Cancer-related
Ascending	Female	87	Grade 2	T3N0M0 Stage II	Cancer-related
Descending	Female	69	Grade 2	T2N0M0 Stage I	Cancer-related
Ascending	Female	86	Grade 3	T3N0M0 Stage II	Not cancer-related
Cecum	Male	66	Grade 2	T3N0M0 Stage II	Unknown
Sigmoid	Male	64	Grade 2	T3N0M0 Stage II	Unknown
Right	Male	62	Grade 2	T3N0M0 Stage II	Not applicable
Colon	Female	67	Grade 2	T3N0M0 Stage II	Cancer-related
Transverse	Female	83	Grade 3	T4N0M0 Stage II	Not cancer-related

A.42 Characterization of Malignancies with Deficient DNA Mismatch Repair Proteins Demonstrating a Loss of MLH1/PMS2 (continued).

Table A.11. Characterization of Malignancies with Deficient DNA Mismatch Repair Proteins Demonstrating a Loss of MLH1/PMS2 (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Cecum	Female	83	Grade 3	T3N0M0 Stage II	Cancer-related
2005- Right colon synchronous malignancy	Female	80	Block 2- Grade 2 Block 6- Grade 1 arising in tubular adenoma	T3N0Mx Stage II T1N0Mx Stage I	Unknown
2007- Right colon Biopsy & Resection			Biopsy Grade 3 Resection Grade 3	not staged T3N0Mx Stage II	Unknown
Synchronous lesions: Block B6- Right / ascending Block B10- Right / ascending distal to 1^0 malignancy	Female	79	Grade 4 Grade 4	T3N0Mx Stage II	Unknown
Synchronous lesions: block #3- Ascending block #7- Transverse	Female	70	Block #3- Grade 3 Block #7- Grade 2	T2N0M0 Stage I	Not applicable

A.43 Characterization of Malignancies with Deficient DNA Mismatch Repair Proteins Demonstrating a Loss of One Repair Protein.

Table A.12. Characterization of Malignancies with Deficient DNA Mismatch Repair Proteins Demonstrating a Loss of One Repair Protein

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Splenic flexure	Female	67	Grade 2	T4N0M0	Cancer-related
Cecum	Male	88	Grade 2	T2N0M0 Stage I	Cancer-related
Right colon	Male	53	Grade 2	T3N0M0 Stage II	Not applicable
Ascending	Female	79	Grade 4	T3N0Mx Stage II	Unknown

A.44 Positive PD-L1 Expression in Colon Resection and Matched Lymph Node.

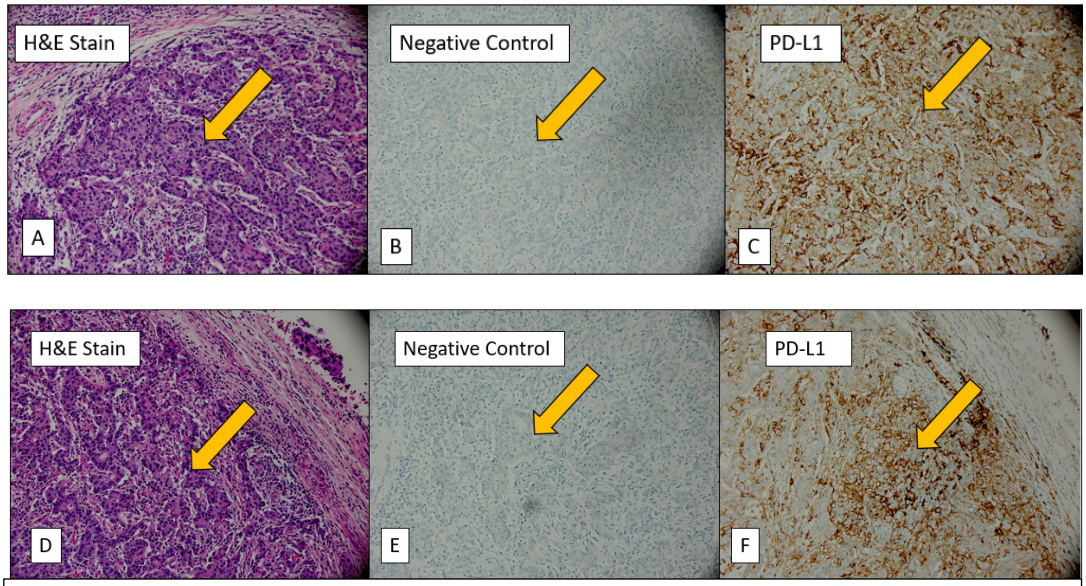

Figure A.11. Positive PD-L1 Expression in Colon Resection and Matched mCRC in Lymph Node: a) Hematoxylin & Eosin stain (arrow denotes) malignant cells, **b)** Rabbit monoclonal negative reagent control **c)** Positive membranous staining in 70-89% of tumor cells, **d)** Hematoxylin & Eosin stained lymph node belonging to the same patient, **e)** Rabbit monoclonal negative reagent control, **f)** Positive membranous staining in 90-100% of tumor cells. All images were taken at 40x magnification.

A.45 Negative PD-L1 Expression in Colon Resection and Matched mCRC in Lymph Node.

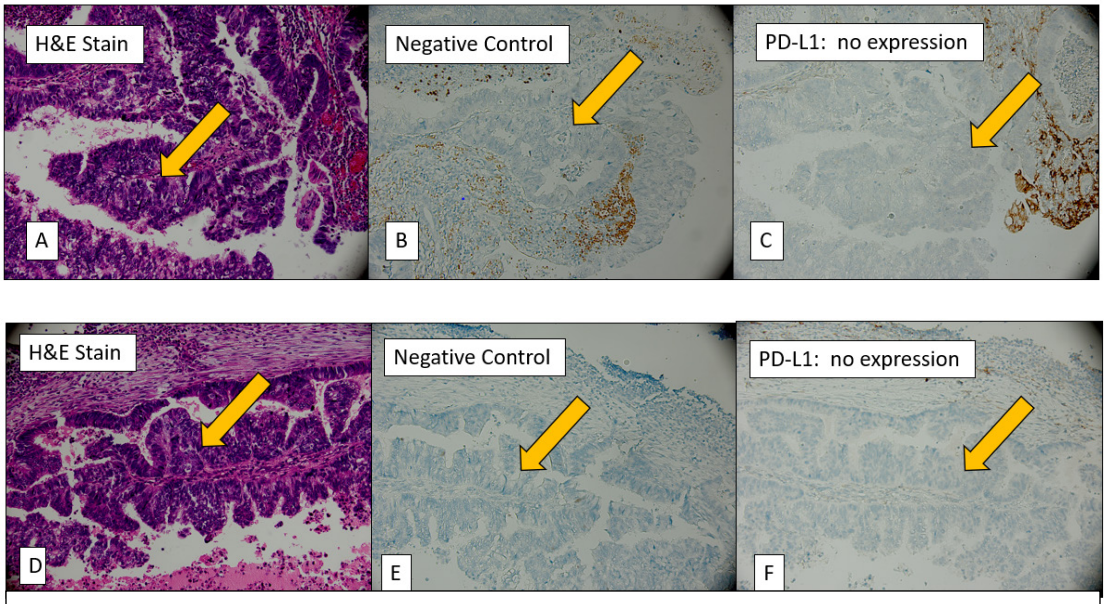

Figure A.12. Negative PD-L1 Expression in Colon Resection and Matched mCRC in Lymph Node: a) Hematoxylin & Eosin stain (arrow denotes) malignant cells, **b)** Rabbit monoclonal negative reagent control with endogenous background staining noted, **c)** Negative membranous staining in tumor cells but positive staining in macrophages in microenvironment (internal positive control) **d)** Hematoxylin & Eosin stained lymph node with mCRC belonging to the same patient, **e)** Rabbit monoclonal negative reagent control, **f)** Negative membranous staining of tumor cells. Staining of tumor-infiltrating immune cells is noted. All images were taken at 40x magnification.

A.46 Characterization of PD-L1 and Clinicopathological Features in Patient Cohort.

Table A.13. Characterization of PD-L1 and Clinicopathological Features in Patient Cohort.
PD-L1 Expression levels varied among matched samples in the cohort. PD-L1 expression was observed more frequently in tumors arising in the sigmoid and proximal colon. Tumors demonstrating the greatest percentage of PD-L1 arose in the cecum.

Score	Patient Demographics			WHO Histological Grade	AJCC Staging	Cause of Death	Characteristics of Matched Samples
1- 9% expression in TC	Source	Gender	Age of Patient				
	Cecum	Male	70	Grade 2	T3N2M1 Stage IV	Cancer-related	Resection and Node MMR Intact
	Sigmoid	Male	49	Grade 2	T3N1M0 Stage III	Unknown	Resection MMR Intact
	Sigmoid	Female	82	Grade 2	T1N0M0 Stage I	Cancer-related	Bx 1-9%, Resection 1-9% expression deficient (loss of MLH1/PMS2) BRAF V600E
	Right colon	Male	83	Grade 2	T3N0Mx Stage II	Cancer-related	Bx <1%; Resection 1-9% MMR deficient (loss of MLH1/PMS2) BRAF V600E
	Splenic flexure	Male	64	Grade 1	T3N0M0 Stage II	Unknown	Resection; MSS BRAF V600E
	Sigmoid	Male	68	Grade 3	T1N0M0 Stage I	Cancer-related	Resection; MSS
	Cecum	Female	83	Grade 3	T3N0M0 Stage II	Cancer-related	Resection; deficient (loss of MLH1/PMS2) BRAF V600E
	Sigmoid & Rectum	Female	70	2005 Sigmoid, grade 2 2006, Rectum Grade 2 2007 Rectum Grade2	T3N2Mx Stage III T3N2Mx Stage III T3N2Mx Stage III	Cancer-related	1-9% expression in resection, no expression in nodes No expression No expression

A.47 Characterization of PD-L1 and Clinicopathological Features in Patient Cohort (continued).

Table A.13. Characterization of PD-L1 and Clinicopathological Features in Patient Cohort (continued)

Score	Patient Demographics			WHO Histological Grade	AJCC Staging	Cause of Death	Notes
10- 29% expression in TC	Source	Sex of Patient	Age of Patient				
	Appendix	Male	60	Grade 2	T1N0M0 Stage I	Not cancer-related	Resection; MSS
	Right Colon	Female	80	2005 malignancy Block 2: Grade 2 Block 6: Grade 1 2007 Bx: grade 3 Resection: Grade 3	 T3N0Mx Stage II T1N0Mx Stage I Not graded T3N0Mx Stage II	Cancer-related	 10-29% expression 1-9% expression 1-9% expression BRAF V600E positive 10-29% expression; BRAF V600E positive

Score	Patient Demographics			WHO Histological Grade	AJCC Staging	Cause of Death	Notes
30-100% expression in TC	Source	Sex of Patient	Age of Patient				
	Cecum	Female	81	Grade 3	T4N2M0 Stage III	Not cancer-related	Bx 30%, colon 75%, nodes 30% All dMMR: LossMLH1/PMS2 BRAF V600E mutation
	Cecum	Female	72	Grade 2	T3N2M0 Stage III	Unknown	Bx 30%, colon 70%, nodes 90% All dMMR: LossMLH1/PMS2

A.48 Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies.

Table A.14. Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies.
Twenty-nine percent of the patients in our cohort had malignancies in which multiple mutations were identified. Malignancies arising in the ascending colon demonstrated the greatest mutational diversity and the frequency of concomitant mutations gradually decreased from the transverse colon to the rectum.

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Staging	Cause of Death	Summary of Mutations:	Treatment Summary:
Male	74	B1- Ileocecal D1- Sigmoid Resection Sigmoid	Block #B1: Grade 2 Block #D1: Grade 2 Resection: Grade 2	TisNxMx Stage 0 TisNxMx Stage 0 T1N0M0 Stage1	Unknown	MMR Status: Proficient / intact. PDL-1 Expression: no expression in TC. BRAF mutation – No mutation identified via IHC or PCR KRAS mutation (PCR)- No mutation detected in any of the samples. PIK3CA mutation (PCR)- No mutation detected in any of the samples.	Colonoscopy Right hemi-colectomy with lymph node dissection. Observation.

A.49 Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued).

Table A.14. Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death	Summary of Mutations:	Treatment Summary:
Gender	Age	Source					
Male	83	#3- Cecum #8- Right	Both blocks, #3 & 8: Grade 2	Block #3 T2N0Mx Stage I Block #8 T1N0Mx Stage I	Unknown	MMR Status: Proficient / intact. PDL-1 Expression: no expression in TC. BRAF mutation – No mutation identified via IHC or PCR KRAS mutation (PCR)- No mutation detected in any of the samples. PIK3CA mutation (PCR)- No mutation detected in any of the samples.	Colonoscopy with biopsy; Right hemi-colectomy with lymph node dissection. Observation.

A.50 Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued).

Table A.14. Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Stage	Cause of Death	Summary of Mutations:	Treatment Summary:
Gender	Age	Source					
Female	70	#3- Ascending #7- Transverse	Block #3- Grade 3 Block #7- Grade 2	Both were staged: T2N0M0 Stage I	NA	MMR Status: deficient; loss of MLH1/PMS2 in both samples. PDL-1 Expression: no expression in TC. BRAF mutation BRAF V600E mutation identified in both samples via IHC and PCR KRAS mutation (PCR)- No mutation detected in either sample. PIK3CA mutation (PCR)- No mutation detected in either sample.	Colonoscopy; Right hemicolectomy with lymph node dissection. Observation.

A.51 Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued).

Table A.14. Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Stage	Cause of Death	Characterization of Mutations:	Treatment Summary:
Gender	Age	Source					
Male	78	Descending	Blocks #3 & 8 Grade 2	T4N2Mx	Cancer-related	MMR Status: Proficient/ intact in both lesions. PDL-1 Expression: Block #3: <1% expression in TC; Block #8: no expression in TC. BRAF mutation – No BRAF V600E mutation identified via IHC or PCR in either block. KRAS mutation (PCR)- Block #3- KRAS mutation G12x identified. Block #8: No mutation detected. PIK3CA mutation (PCR)- Block #3: No mutation detected. Block #8: PIK3CA mutation E545x detected.	Colonoscopy with biopsies; Left hemicolectomy; low anterior resection with ileostomy

A.52 Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies.

Table A.14. Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death	Summary of Mutations:	Treatment Summary:
Gender	Age	Source					
Female	74	B3- Sigmoid / ulcerating lesion B9- Sigmoid/ proximal to original bx site	Grade 3 Grade 2	T2N0 Mx	Unknown	MMR Status: Proficient/ intact in both blocks. PDL-1 Expression: no expression in either sample. BRAF mutation – No mutation identified in either sample via IHC and PCR KRAS mutation (PCR)- No mutation detected in B3; G12X mutation detected in B9. PIK3CA mutation (PCR)- No mutation detected in either sample.	Not available due to registry changes.

A.53 Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued).

Table A.14. Diversity of Mutations and Clinicopathological Features in Patients with Synchronous Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death	Summary of Mutations:	Treatment Summary:
Gender	**Age**	**Source**					
Female	79	B6- Right / ascending B10- Right / ascending distal to 1^0 malignancy	Grade 4 Grade 4	T3N0Mx T3N0Mx	Not-cancer-related	Blocks #B6 and #B10: MSI- Status: B6- Deficient / loss MLH1/PMS2 B10- Deficient / loss MLH1 PDL-1 Expression: no expression in TC. BRAF V600E mutation – B6- No mutation identified via IHC or PCR B10- yielded invalid results via PCR; IHC stain was negative. KRAS mutation (PCR)- No mutation detected in B6; B10 yielded invalid results. PIK3CA mutation (PCR)- E545x mutation detected in both samples.	Unavailable due to registry changes.

A.54 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies.

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies.
Recurrent malignancies demonstrated mutations that were similar to the original malignancy.

Patient Demographics			WHO Grade	AJCC Staging	Cause of Death	Characterization of Mutations:	Treatment Summary:
Gender	Age	Source					
Female	90	Rectum	Bx: Grade 2 Resection: Grade 2	Not staged T3NxMx Stage II	Cancer-related	Biopsy and Resection: MMR protein markers (IHC): intact in bx and resection. PDL-1 Expression- no expression in bx or resection. BRAF mutation (IHC)- no expression in bx or resection. BRAF/ NRAS mutation (PCR)- no mutation detected in bx or resection. KRAS mutation (PCR)- G12X mutation detected in bx and resection. PIK3CA mutation (PCR)- no mutation detected in bx or resection.	Biopsy, local tumor excision and fulguration rectal lesion. Radiation Perirectal Region

A.55 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued).

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:	Treatment Summary:
Female	90	Recurrence: Rectum	Bx:	T3N0M0 Stage II	Cancer-related	MSI- Status: stable PDL-1 Expression- no expression in tumor cells. BRAF mutation (IHC)- no expression BRAF/ NRAS mutation (PCR)- no mutation identified. KRAS mutation (PCR)- G12X mutation detected. PIK3CA mutation (PCR)- no mutation detected.	Unknown

A.56 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies.

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Stage	Cause of Death	Characterization of Mutations:	Treatment Summary:
Female	80	Right Colon	Block #2- Grade 2 Block #6- Grade 1 arising in tubular adenoma	T3N0Mx Stage II T1N0Mx Stage I	Cancer-related	MMR status: deficient; both samples demonstrated a loss of MLH1/PMS2. PDL-1 Expression: Block 2: 10-29% expression in TC. Block 6: 1-9% expression in TC. BRAF (V600E) mutation -both samples were positive via IHC and PCR KRAS mutation (PCR)- no mutation detected in either sample. PIK3CA mutation (PCR)- Block 2: H1047X and E545X mutations detected. Block 6: No mutation detected.	Not avail

A.57 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued).

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued)

Patient Demographics			WHO Grade	AJCC Stage	Cause of Death	Characterization of Mutations:	Treatment Summary:
Female	80	Recurrent, Right colon Biopsy & Resection	Biopsy: Grade 3 Resection: Grade 3	Biopsy: not staged Resection: T3N0Mx Stage II	Cancer-related	MMR status: deficient; both samples demonstrated a loss of MLH1/PMS2. PDL-1 Expression: Biopsy: 1-9% expression in TC. Resection: 10-29% expression in TC. BRAF mutation: Biopsy: BRAFV600E positive IHC; Resection: BRAF V600E mutation identified via IHC and PCR KRAS mutation (PCR)- no mutation detected . PIK3CA mutation (PCR)- no mutation detected.	Not avail.

A.58 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued).

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued)

Patient Demographics			*WHO Grade*	AJCC Stage	Cause of Death	Characterization of Mutations:	Treatment Summary:
Female	73	Right colon Recurrent sample QNS	Grade 2	T3N0M0 Stage II	Unknown	MMR - Status: proficient; intact in resection PDL-1 Expression: <1% in TC, lots in IC in microenvironment. BRAF mutation (IHC)- no expression BRAF/ NRAS mutation (PCR)- no mutation identified. KRAS mutation (PCR)- no mutation detected PIK3CA mutation (PCR)-no mutation detected.	Colonoscopy, ilecolectomy. Surgical resection of anastomotic site recurrence. Patient chose observation.

A.59 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued).

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued)

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:	Treatment Summary:
Gender	Age	Source					
Female	70	Sigmoid	Grade 2	T3N2Mx Stage III	Cancer-related	MMR Status: Proficient / intact. PDL-1 Expression: Colon: 1-9% expression in TC. Nodes: no expression in TC. BRAF mutation – no mutation identified via IHC or PCR KRAS mutation (PCR)- G13x mutation detected in both samples. PIK3CA mutation (PCR)- No mutation detected in either sample.	Low Anterior Resection. Radiation. 5FU + leucovorin Folfox-6, Avastin Folfiri Vectibix

A.60 Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued).

Table A.15. Diversity of Mutations and Clinicopathological Features in Patients with Recurrent Malignancies (continued)

Patient Demographics Gender	Age	Source	WHO Histologic Grade	AJCC Staging	Cause of Death	Characterization of Mutations:	Treatment Summary:
		Recurrence: Rectum	Grade 2	T3N2Mx Stage III	Cancer-related	MMR Status: Proficient / intact. PDL-1 Expression: No expression in TC in rectum or sacrum. BRAF mutation – no mutation identified via IHC or PCR KRAS mutation (PCR)- G13x mutation detected. PIK3CA mutation (PCR)- No mutation detected.	Not available.
		Recurrence : Rectum	Grade 2	Refers to original staging		MMR Status: Proficient / intact. KRAS mutation: G13x mutation detected.	Not available.

A.61 Characterization of Chemoresistant Malignancies.

Table A.16. Characterization of Chemoresistant Malignancies.
Chemoresistance was presumed based on multiple courses of cytotoxic therapies and treatment notes. Malignancies arising in the rectum were the most frequent source of chemoresistant tumors in our cohort, however, this finding is not generalizable due to the small number of samples in this category.

Patient Demographics			**WHO Grade**	**AJCC Stage**	**Cause of Death**	**Characterization of Mutations:**	**Treatment Summary:**
Male	46	Rectum	Grade 3	T2N M0 Stage III	Unknown	MMR IHC: Intact in Bx, resection and nodes. PDL-1 Expression: no expression in bx, resection or nodes. BRAF mutation (IHC)- no expression in bx, resection or nodes. BRAF/ NRAS mutation (PCR)- no mutation identified in bx; resection KRAS mutation (PCR)- no mutation identified in bx; resection PIK3CA mutation (PCR)- no mutation identified in bx; resection.	**Surgical Intervention:** Colonscopy w/ biopsies and Low anterior resection **Chemotherapy and Radiation**: Folfox , Avastin; radiation Folfiri , Avastin, Radiation Folfiri, Eribitux Radiation - thoracic spine. Samarium Erbitux, Xeloda

A.62 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (Continued).

Patient Demographics			WHO Grade	AJCC Stage	Cause of Death	Characterization of Mutations:	Treatment Summary:
Female	65	Rectum	Grade 2	T3 N1 M0 Stage III	Cancer-related	MMR proteins (IHC): Intact in bx, resection and Nodes PDL-1 Expression- no expression in bx, resection or nodes. BRAF mutation (IHC)- no expression in bx, resection or nodes. BRAF/ NRAS mutation (PCR)- no mutation detected in bx; resection and nodes yielded invalid results. KRAS mutation (PCR)- no mutation detected in bx; resection and nodes yielded invalid results. PIK3CA mutation (PCR)- no mutation detected in bx or resection; nodes yielded invalid results.	Surgical Intervention: Colonoscopy & biopsies Low Anterior Resection Chemotherapy and Radiation: 5FU & leucorvorin Folfox-6 & Avastin Radioactive sir-spheres

A.63 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (continued).

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Stage	Cause of Death	Summary of Mutations:	Treatment Summary:
Female	45	sigmoid	Grade 2	T3N1M0 Stage III	Unknown	MMR proteins: Intact in bx, resection and nodes. PDL-1 Expression- no expression in bx, resection or nodes. BRAF mutation (IHC)- no expression in bx, resection or nodes. BRAF/ NRAS mutation (PCR)- no mutation identified in resection. KRAS mutation (PCR)- G13X identified in colon resection but not in nodes. PIK3CA mutation (PCR)- no mutation identified.	Surgical Intervention: Colonoscopy Segmental resection Chemotherapy and radiotherapy: Folfox Radio-frequency ablation: liver mets, Folfox- 4 Xeloda / Avastin

A.64 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (continued).

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Stage	Cause of Death	Summary of Mutations:	Treatment Summary:
Male	53	Ascending	Grade 2	T3N2M1 Stage IV	Cancer -related	MMR protein markers (IHC): Intact in resection and nodes. PDL-1 Expression- no expression in resection or nodes. BRAF mutation (IHC)- no expression in resection or nodes. BRAF/ NRAS mutation (PCR)- no mutation identified in resection or nodes. KRAS mutation (PCR)- A146X identified in resection and nodes . PIK3CA mutation (PCR)- E545X mutation identified in nodes but not in colon.	Surgical Intervention: Liver biopsy Colonoscopy with biopsies Right hemicolectomy Chemotherapy and Radiotherapy: Folfox-7 + Avastin Folfiri + Avastin Yttrium-90 radioactive sir-spheres

A.65 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (continued).

Patient Demographics Gender	Age	Source	WHO Grade	AJCC Stage	Cause of Death	Characterization of Mutations:	Treatment Summary:
Female	70	Overlapping lesion	Grade 2	T3 N1 M0 Stage III	Deceased	MMR proteins (IHC): Intact in bx, resection and nodes. PDL-1 Expression- no expression in bx, resection or nodes. BRAF mutation (IHC)- no expression in bx, resection or nodes. BRAF/ NRAS mutation (PCR)- no mutation detected in bx, resection or nodes. KRAS mutation (PCR)- no mutation detected in bx, resection or nodes. PIK3CA mutation (PCR)- no mutation detected in bx, resection or nodes.	Surgical Intervention: Biopsy Ileocolectomy Chemotherapy Regimen: Folfox Folfiri + Avastin 5FU, Irinotecan, Avastin Xeloda

A.66 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (continued).

Patient Demographics			**Grade**	**AJCC Stage**	**of Death**	**Characterization of Mutations:**	**Treatment Summary:**
Gender	**Age**	**Source**					
Female	69	Transverse	Grade 3	T3 N0 M0 Stage II	Not cancer-related	MMR protein markers (IHC): Intact. PDL-1 Expression- No expression. BRAF mutation (IHC)- no expression present. BRAF/ NRAS mutation (PCR)- no mutation detected. KRAS mutation (PCR)- G12X mutation detected. PIK3CA mutation (PCR)- no mutation detected.	Surgical intervention: Colonoscopy Colon Resection Chemotherapeutic regimen: 5FU and Leucovorin Observation

A.67 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (continued).

Patient Demographics Gender	Age	Source	**WHO Grade**	**AJCC Stage**	**Cause of Death**	**Characterization of Mutations:**	**Treatment Summary:**
Male	53	Right colon	Grade 2	T3 N0 M0 Stage II	Not applicable	MMR status: deficient, loss of PMS2 PDL-1 Expression- No expression. BRAF (IHC)- no expression. BRAF/ NRAS mutation (PCR)- NRAS Q61x mutation identified. KRAS mutation (PCR)- no mutation detected. PIK3CA mutation (PCR)- no mutation detected.	Surgical Intervention: Colonoscopy/ EGD Bx Colectomy Resection of liver mets Chemotherapeutic Regimen: Observation Folfox + Avastin Folfiri

A.68 Characterization of Chemoresistant Malignancies (continued).

Table A.16. Characterization of Chemoresistant Malignancies (continued).

Demographics Gender	Age	Source	Grade	AJCC Stage	Death	Characterization of Mutations:	Treatment Summary:
Male	51	Colon	Grade 2	T3N0M0 Stage II	Cancer-related	MMR - Status: intact in resection PDL-1 Expression- <1% expression in tumor cells. BRAF mutation (IHC)- no expression BRAF/ NRAS mutation (PCR)- no mutation detected. KRAS mutation (PCR)- no mutation detected. PIK3CA mutation (PCR)- no mutation detected.	Surgical Intervention: appendectomy and ileocolectomy Chemotherapeutic Regimen: Xeloda Folfox Folfiri, Avastin Irinotecan, Erbitux Xelox, Avastin

A.69 Clinicopathological Features of Malignancies in Which No Mutations Were Detected.

Table A.17. Clinicopathological Features of Malignancies in Which No Mutations Were Detected. No mutations were detected in a subset of samples in this study. Of these, a majority of the primary tumors arose in the rectum and belonged to males.

Patient Demographics			**WHO Histologic Grade**	**AJCC Staging**	**Cause of Death**
Source	Gender	Age			
Rectum	Male	46	Grade 3	T2N0M0	Unknown
Hepatic Flexure	Female	65	Grade 2	T3N0M0	Cancer related
Rectum	Male	78			
Rectum	Female	65	Grade 2	T3N1M0	Cancer-related
Rectum	Male	78	Grade 3	T3N2M0	Cancer-related
Overlapping Lesion	Female	70	Grade 2	T3N1M0	Cancer-related
Rectum	Male	83	Grade 3	T3N2M0	Unknown
Rectum	Male	68	Grade 2	T1N0M0	Unknown
Rectum	Male	76	Grade 2	T3N0M0	Not applicable
Sigmoid	Male	78	Grade 2	T3N0M0	Cancer-related
Transverse	Male	82	Grade 2	T4N0M0	Cancer-related
Male	Rectum	55	Grade 2	T2N0M0	Unknown
Male	Rectum	56	Grade2	T3NxMx	Cancer-related

A.70 Clinicopathological Features of Malignancies in Which No Mutations Were Detected (continued).

Table A.17. Clinicopathological Features of Malignancies in Which No Mutations Were Detected (continued).

Patient Demographics			WHO Histologic Grade	AJCC Staging	Cause of Death
Source	**Gender**	**Age**			
Male	Sigmoid	56	Grade 2	T2N0M0	Not cancer-related
Rectum	Male	33	Grade 2	T2N0M0	Unknown
Rectum	Female	65	Grade 2	T3N0M0	Cancer-related
Transverse	Male	72	Grade 2	T3N0M0	Not cancer-related
Ascending	Female	66	Grade 2	T1N0M0	Unknown
Sigmoid	Male	80	Grade 2	T3N0M0	
Cecum	Male	83	Grade 3	T1N0M0	Cancer-related
Hepatic flexure	Female	66	Grade 2	T3N0M0	
Right colon	Male	68	Grade 2	T1N0M0	Not cancer-related
Rectum	Male	73	Grade 2	T3N0M0	Unknown
Hepatic flexure	Male	52	Grade 2	T3N0M0	Unknown
Sigmoid	Male	58	Grade 1	T1N0M0	Not cancer-related
Sigmoid	Male	74	Grade 2 for synch malig x2 and resection	Synch= TisNxMx Resection= T1N0Mx	Unknown
Cecum	Male	83	Grade 2	T2N0M0	Unknown

A.71 Clinicopathological Features of Patients Who Died From Cancer-Related Causes.

Table A.18. Clinicopathological Features of Patients Who Died From Cancer-Related Causes. Thirty-seven percent of the patients in this cohort expired due to cancer-related causes. Interestingly, 57% of the patients that passed away were originally diagnosed with localized disease (white rows) or synchronous malignancies (green rows) vs. those originally diagnosed with metastatic disease (yellow rows). Over 40% of the patients who passed away from CRC had primary tumors arising in the proximal colon.

Summary of Patients With Cancer-Related Cause of Death			
Sex of Patient	**Age of Patient** (years)	**Location of Originating Malignancy**	**Classification of Tumor (Right vs. Left Colon)**
Male	69	Sigmoid	Left
Male	70	Cecum	Right
Female	66	Transverse	Transverse
Male	78	Rectum	Rectum
Female	65	Rectum	rectum
Female	71	Cecum	Right
Male	53	Ascending	Right
Male	78	Rectum	Rectum
Female	70	Overlapping lesion	N/A
Male	68	Rectum	Rectum
Female	74	Sigmoid	Left
Female	66	Transverse	Transverse
Female	66	Cecum	Right
Female	60	Descending	Left
Male	81	Ileocecal	Right
Male	78	Sigmoid	Left
Female	82	Sigmoid	Left
Male	82	Transverse	Transverse
Male	83	Right colon	Right
Male	68	Sigmoid	Left
Male	56	Hepatic flexure	Right
Female	87	Ascending	Right
Male	56	Rectum	Rectum
Female	86	Ascending	Right
Female	69	Descending	Left
Female	76	Transverse	Transverse
Female	65	Rectum	Rectum
Male	84	Hepatic flexure	Right
Male	76	Cecum	Right
Male	64	Transverse	Transverse
Female	78	Ascending	Right
male	75	Transverse	Transverse
Female	67	Splenic flexure	Left
Male	88	Cecum	Right
Male	83	Cecum	Right
Female	66	Hepatic flexure	Right
Female	67	Right colon	Right
Male	89	Splenic flexure	Left
Male	51	Colon, NOS	NA
Female	83	Cecum	Right
Female	90	Rectum	Rectum
Female	80	Right colon	Right
Female	70	Rectum	Sigmoid & Rectum
Male	78	Descending	Left

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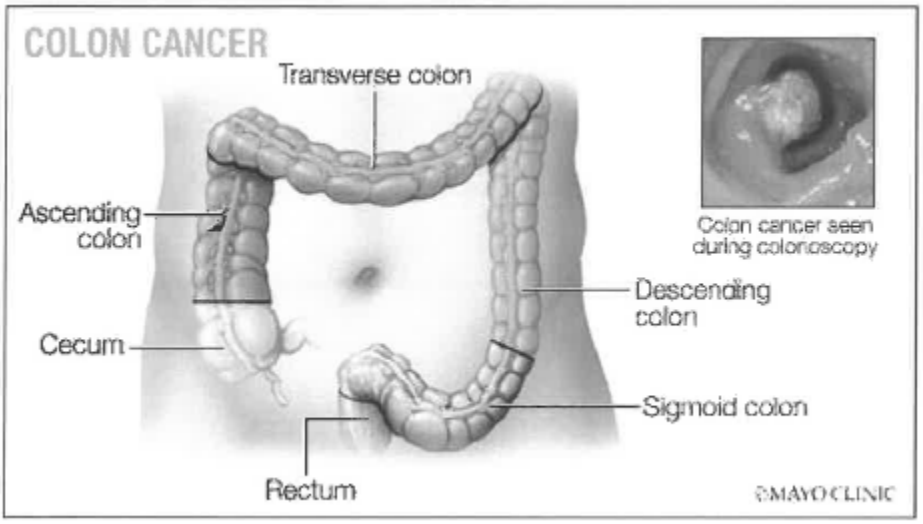
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