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Novel Non-Invasive Strategies Using Genetic Biomarkers for Early Detection of Colorectal Cancer

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ABSTRACT

Among individuals aged between 20 years and 49 years, colorectal cancer (CRC) is one of the leading causes of death. This is partly due to the fact that CRC is not diagnosed earlier because symptoms do not showcase until later stages—a hallmark of CRC. The gold standard testing for CRC continues to be the colonoscopy, a test that discourages patients because of its invasive nature. In light of this problem, this research paper methodically reviews previously published papers and current/ongoing studies to explore a testing process for CRC that uses genetic biomarkers (mutated APC, KRAS, and TP53 genes and methylated DNA) in the stool samples. The genetic markers chosen were based on the major genes involved in the colorectal carcinogenesis pathway. Testing these genetic biomarkers in combination has shown promise by increasing sensitivity and efficiency of CRC screening by using technologies, including next generation sequencing, polymerase chain reaction (PCR) and methylation-specific PCR. Such non-invasive testing methods can encourage patient compliance and help combat the growing death rate. In the future, utilizing genetic biomarkers should become more widespread, increasing CRC diagnosis rates. Additionally, individuals should continue to pay attention to unusual symptoms, reach out to medical professionals, and take a more proactive stance in their health. The end of this paper explores several ongoing or completed clinical trials that use various types of biomarkers (which are not limited to genetic biomarkers), showcasing the urgency of this issue.

Introduction

CRC is considered the third most prevalent cancer and second leading cause of death worldwide. More than 1.9 million new cases and 930,000 deaths due to colorectal cancer were estimated to have occurred in 2020. By 2040, it is expected that the number of new cases will increase to 3.2 million per year and 1.6 million deaths per year (World Health Organization, 2023).

Early detection is extremely important for cancer treatment because it can increase treatment effectiveness and improves prognosis. While there are numerous invasive and non-invasive screening methods for CRC, the "gold standard screening" continues to be the colonoscopy (Godman, 2024). Because a colonoscopy is expensive to perform, requires advanced technology, and invasive preparation beforehand, it has low patient compliance which hinders prevention or early treatment of CRC in at-risk populations and underserved communities. The current use of non-invasive tests, such as the fecal immunochemical test (FIT), are not as sensitive in detecting CRC in stage one (53%) and early adenomas (27%) (Han et al., 2019).

This places an increasing burden on asymptomatic cases, which is a hallmark of CRC. Patients in earlier stages do not have symptoms, or the symptoms may resemble other diseases, making it harder to diagnose (Sullivan & Lewis, 2023). More strikingly, a study has estimated that by 2030, CRC will be the leading cause of death in individuals aged between 20 years and 49 years of age (Rahib et al., 2021). Part of the issue can be associated with typical CRC screening done starting from the age of 45, which misses the young adult population window (Sullivan & Lewis, 2023).

In light of this growing issue, it is of utmost importance that non-invasive screening methods using molecular biomarkers are developed, especially for individuals reluctant to undergo colonoscopies (Han et al., 2019). Because non-invasive stool tests are more appealing for patient adherence, developing accurate stool tests using biomarkers of colorectal carcinogenesis may be a solution. Analysis of gene mutations in APC, KRAS, and TP53 and methylation biomarkers from fecal DNA using technologies, such as polymerase chain reaction (PCR), methylation-specific PCR, and next generation sequencing, are promising strategies. This paper explores novel combination gene testing processes in an attempt to identify new, early detection methods for CRC.

Methodology

The objective of this study is to explore new testing processes for CRC diagnosis. This study focuses on stool tests that can be performed periodically in hopes of diagnosing CRC earlier, especially in younger adults (younger than 50 years of age). This paper explains what CRC is; existing, established CRC screening procedures; various pathways to colorectal carcinogenesis; studies that test mutations in APC, KRAS, and TP53 and methylation biomarkers; studies that use said mutations, and other markers, in combination with certain technologies for early detection; and current clinical trials. This study is a secondary literature review of previously published research articles from PubMed, NCBI, NEJM, current clinical trials (clinicaltrials.gov), international sources and existing observations regarding the relationship between CRC diagnoses and said biomarkers. This process occurs primarily by interpreting primary research studies and is guided with the help of two professors. This study does not perform, organize, or use any physical tools or materials. As a result, no ethical considerations were made during the length of this study. Research biases are alleviated by using a plethora of online resources, utilizing cross-referencing methods, and consulting with two guiding professors as needed.

CRC: Symptoms, Causes, and Family History Incidence

Colorectal cancer is defined as the occurrence of uncontrolled proliferation of cells in the colon and rectum. CRC begins by the growth of polyps, which is growth in the mucosa of the colon and/or rectum. If certain polyps are not removed, they can become cancerous. For example, adenomatous polyps, sessile serrated polyps, and traditional serrated polyps are pre-cancerous. On the other hand, hyperplastic and inflammatory polyps are benign. The size of a polyp, the number, and the histology can increase an individual's chances of developing CRC (American Cancer Society, 2020). If pre-cancerous polyps are not removed, they can grow outward from the mucosa and into other cell walls, blood vessels, and lymph nodes. If left untreated, the tumor can metastasize to the liver, lungs, brain, and the pelvic region. The most common CRC are adenocarcinomas and make about 90% to 95% of all large bowel tumors (National Cancer Institute, n.d.).

CRC can be hard to diagnose in its earlier stages because it doesn't always cause symptoms. That is why it is extremely important that individuals undergo regular screening so CRC can be diagnosed earlier and polyps can be removed. Symptoms of CRC can vary across patients; however, these symptoms are regularly reported: changes in bowel habits (having frequent diarrhea or constipation); incomplete bowel movement; traces of blood in the stool; bloating and abdominal pain; and rapid weight loss (Centers for Disease Control and Prevention, 2024).

CRC is caused by mutations in an individual's DNA. The environment one lives in and the lifestyle may trigger such mutations or worsen the effect of existing mutations. Consumption of highly processed foods, with inadequate amounts of fruit and vegetables in the diet, having a sedentary lifestyle, smoking, high alcohol consumption, and obesity can increase an individual's chances of developing CRC. Age also plays a large role in developing CRC, with most cases occurring at the age of 50 (World Health Organization, 2023). However, as previously discussed, CRC diagnosis and deaths have been rising steadily among adults younger than 50 since the 1990s (National Cancer Institute, 2020).

Family history of CRC plays a large part in the development of the disease. For example, individuals with a family history of Lynch Syndrome and familial adenomatous polyposis (caused by APC germline mutations) or had polyps previously removed are more likely to develop CRC. According to a recent study, an individual's risk of developing CRC increases with every first degree relative (FDR) affected. In fact, having one affected FDR increases the risk by two-fold, even with no family history (FH). Notably, a diagnosis of CRC in an FDR before the age of 50 years increases the risk of developing CRC for that individual by three-fold or more. Additionally, the study concludes that an individual's risk of developing CRC is 75% greater if the individual has one or more affected second degree relative but no FH. The rate is lower if the individual has only one affected third degree relative (Lowery et al., 2016). The study concluded that the most important contributor to an individual developing CRC is FDR incidence; however, having one affected FDR, in combination with a second or third degree relative, doubles the risk (Taylor et al., 2010).

In conclusion, screening for CRC is extremely important in combating the disease. Its asymptomatic facade in its early stages, its incidence rate in individuals less than 50 years, and its increasing risk with family history places an urgency for more research about early detection.

Current CRC Screening Tests: Invasive and Non-Invasive

Current screening methods for CRC can be largely broken down into invasive and non-invasive testing. Screening methods, such as colonoscopies, sigmoidoscopies, and the colon capsule, can be considered invasive because they involve prepping or cleansing the patient in advance and use instruments that are inserted into the patient's body. On the other hand, a computed tomography (CT) colonography, stool tests, such as guaiac-based, immunochemical, and multi-target, and the methylated septin 9 blood test are non-invasive (National Cancer Institute, 2021) as shown in Figure 1.

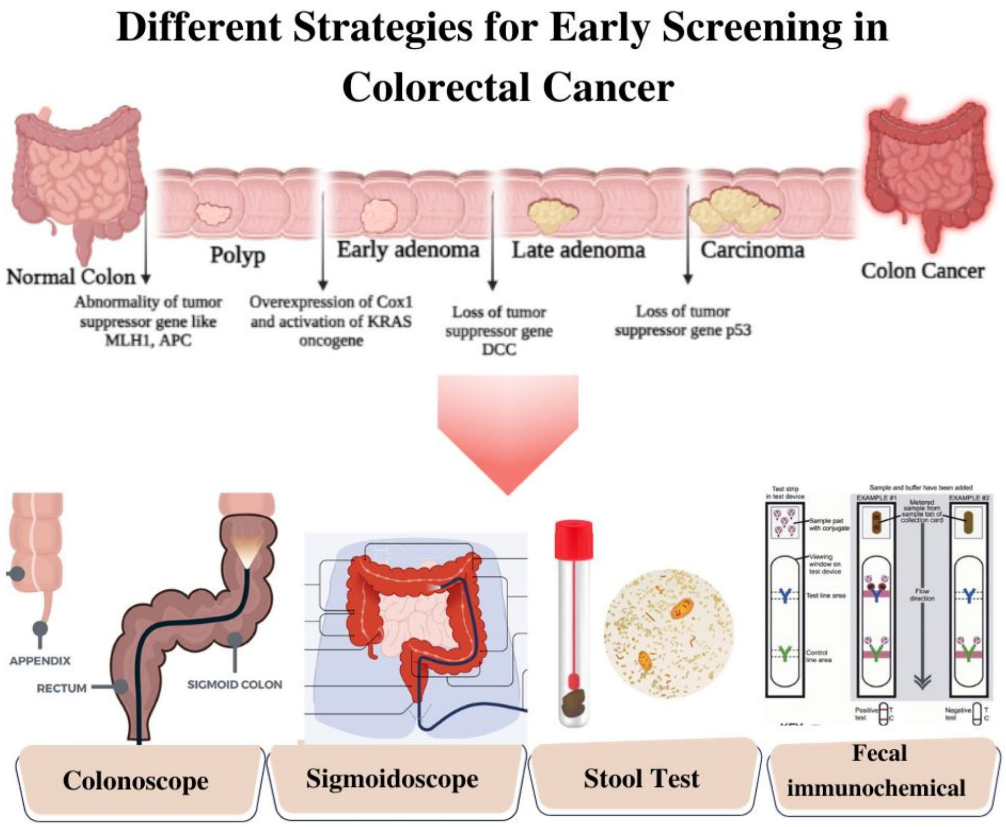

Figure 1. Simplified Illustration of Various Screening Methods for CRC.Source: Beniwal et al., 2023 Description: The upper half of the image illustrates the progression of CRC: a polyp, early adenoma, late adenoma, and carcinoma. The development and changes of certain genes, including its mutation, overexpression, and loss are shown in tally with the progression of CRC. The bottom half of the image exemplifies a few regularly used screening tests, including a colonoscopy, sigmoidoscopy, and stool test (fecal immunochemical test) in left to right order. Additionally, the anus, rectum, and sigmoid colon are labeled on the bottom left diagram.

Considered the gold standard screening, a colonoscopy can identify 95% of CRC cases. The procedure involves inserting a colonoscope, which has a camera attached to a long tube, into the rectum. Before the procedure, the patient must undergo a lengthy process to cleanse their gut. During the procedure, they must be sedated. Polyps can be removed during the procedure and tested if they are pre-cancerous, and allows future recommendations based on the outcome. However, because of the nature of this test, it can be expensive to perform, may result in complications for at-risk patients, and is not always easy to perform (Godman, 2024). A colonoscopy is recommended every 10 years or earlier for at-risk patients (Gude et al., 2023). A similar procedure and instrument, a sigmoidoscope, is used for a sigmoidoscopy. However, only the sigmoid colon needs to be cleared and evaluated, and the patient does not need to be sedated. This test is recommended every 5 to 10 years (National Cancer Institute, 2021).

The colon capsule, also known as the capsule endoscopy, requires the patient to swallow a disposable camera that "takes thousands of pictures" of the digestive tract. This testing process can be used to detect polyps and CRC. To undergo this procedure, the patient must cleanse their bowel. Once swallowed, the capsule will remain in the body for a few hours or days and will be defecated after. Once all the pictures are taken, a professional will review them and recommend further treatment or procedures. A few risks that may be associated with this procedure include discomfort or blocking of the digestive tract. However, these risks are higher "in patients who have a stricture, such as those who have a tumor, Crohn's disease, or have had surgery in the area previously" (Mayo Clinic, 2023).

A CT colonography, also called a virtual colonoscopy, utilizes a CT scanner to take two-dimensional pictures and assembles them into a three-dimensional view of the colon to view its structure, polyps, and abnormal growths. The procedure requires the patient to thoroughly cleanse the bowel (National Cancer Institute, 2021). If polyps or abnormal growths are identified, the patient is then recommended for a colonoscopy. This method causes the patient to have significant exposure to radiation, which is a major drawback (Gude et al., 2023). A study led by Pickhardt (2012) also reveals that a CT colonography may miss important polyps. In this respect, novel non-invasive strategies are being developed that utilizes genetic biomarkers based on early genetic changes in the development of CRC.

The Fecal Occult Blood Test (FOBT) analyzes macroscopically invisible blood in the stool. A guaiac-based test (gFOBT) is one of two types of FOBT that utilizes guaiac to detect a component of hemoglobin (known as heme) in the stool. If heme is found in the stool, the paper turns blue due to an oxidative reaction (Chung et al., 2022). Because certain foods (such as red meat) contain heme, it is advised that the patient stays away from such foods before the test (National Cancer Institute, 2021). The second type of FOBT is the fecal immunochemical test (FIT), also known as an immunochemical FOBT. FIT identifies hemoglobin in the stool by seeing if monoclonal or polyclonal antibodies bind to intact globin components of hemoglobin (Allison et al., 2014). The FIT test identifies CRC more successfully by reducing false-positive results (Shapiro et al., 2017), requiring less stool samples (between one and two), and has no food restrictions, making it more appealing than gFOBT (National Cancer Institute, 2021). Both FOBT tests are recommended once a year (Godman, 2024). If either test is positive, the patient is recommended to undergo a colonoscopy.

The multi-target stool test (mt-sDNA), also known as a FIT-DNA test, commercially available as Cologuard, uses a single stool sample and does not have any food restrictions. The test identifies 10 biomarkers that are associated with colorectal carcinogenesis, including gene mutations (KRAS), gene methylation (BMP3 and NDRG4) (Fatemi et al., 2022), and hemoglobin protein (Berger et al., 2016). If the test is positive, the patient is recommended to undergo a colonoscopy or further testing. *Harvard Health Publishing* adds that mt-sDNA is the most sensitive of the existing three tests, which identifies 92% of CRC and 42% of advanced polyps cases with a false-positive rate of about 13%

(Godman, 2024). This may be due to the fact that mt-sDNA utilizes several biomarkers, in combination. The mt-sDNA test is recommended once every three years.

The last type of test that to be discussed is a blood test. This test is commercially available as Epi proColon. It tests methylated septin 9 (SEPT9), which is known to be methylated early in CRC development. The septin protein family is known to be involved in cell cytokinesis during cell division, and plays an important role as a tumor suppressor gene (MedlinePlus, 2024). The test is recommended for asymptomatic older adults (50+) who cannot undergo traditional screening or who have not previously been screened for CRC (National Cancer Institute, 2021). The results and information about this trial (NCT00855348) can be found in Table 1.

The testing methods discussed in this section are those widely used by medical professionals worldwide or are approved by the Federal Drug Administration. There are a few CRC testing methods that were not discussed.

Colorectal Carcinogenesis Pathways

CRC is known to contain one of the highest mutational burdens of all cancers (The Cancer Genome Atlas Network, 2012). Coupled with the fact that CRC can take a few years to decades to form, it opens questions about developing testing processes that can detect such mutations during the early stages of the colorectal carcinogenesis pathway. Colorectal tumors have multiple progression pathways, including CIN, MSI, and serrated neoplasia (Nguyen et al., 2020). Understanding the major gene mutations, methylations, and signaling pathways associated with colorectal carcinogenesis can guide research towards new testing processes for CRC early detection based on predominant biomarkers. Although there are other pathways, several other processes, proteins and gene mutations involved in CRC development, they are beyond the scope of this paper and will not be discussed.

The chromosomal instability pathway (CIN), making up almost 65% to 70% of all sporadic colorectal tumors, are caused by chromosomal changes. The CIN pathway is initiated by APC gene mutations, which has been identified in nearly 80% of all CIN tumors. Mutations in the APC gene can result in β-catenin stabilization, which acts as a proto-oncogene if not broken down by the APC & β-catenin destruction complex. This process results in the activation of the Wnt signaling pathway, allowing β-catenin to enter the cell's nucleus, bind to TCF, produce a transcription activation complex, and allow for the transcription of genes, such as MYC and Cyclin D1 (Shankar, 2022). Further mutations, including the oncogene KRAS which is found in nearly 40% of colorectal tumors, encourages new growth factor pathways (such as ERK) and molecules to open and proliferate. TP53 gene mutation and 18q loss of heterozygosity (LOH) occur in the final stages of CRC. These mutations are found in 40% and 70%, respectively, of advanced CRC tumors (Nguyen et al., 2020).

The microsatellite instability pathway (MSI) occurs in 15% of sporadic colorectal tumors and mostly those who have Lynch Syndrome. MSI is characterized by mutations in somatic DNA base pairs, such as the mismatch repair (MMR) genes (ex: MLH1), which leads to the instability of DNA microsatellite areas. With increasing errors in the microsatellite region, it makes it harder for DNA polymerase to repair, resulting in frameshift mutations. Because of the MSI phenotype, the process is irreversible and the microsatellite region accumulates more mutations. Most MSI tumors showcase mutations in ^{V600E}BRAF and TGFB receptor-2 gene and hypermethylation at the regulatory genes, including CIMP (CpG island methylator phenotype) and MLH1. Also, 35%-50% of MSI tumors have mutations in the APC gene and TP53, which is less than that found in CIN tumors. While the CIN pathway takes a decade(s) to develop, MSI requires only a few years. There are testing panels in place that test for microsatellite markers, PCR tests for MSI, and immunohistochemical tests for MMR proteins that can help determine if an individual is MSI-high or MSS (microsatellite stability) (Nguyen et al., 2020).

The serrated neoplasia pathway includes the development of serrated polyps, which is a group of polyps that include hyperplastic polyps, sessile serrated adenomas, and/or traditionally serrated polyps. It is estimated that 15% of serrated polyps progress to CRC. The hallmark of the serrated neoplasia pathway is the mutation of the ^{V600E}BRAF gene, leading to the conclusion that this is the alternate pathway of CRC (although much is still not known about this pathway). After the BRAF mutation, the pathway splits into two: the first merges with the MSI pathway, resulting in

MMR mutations and the MSI phenotype; the second undergoes TP53 gene mutations, several oncogenic pathways (Wnt pathway and TGFB signaling), and is MSS (Nguyen et al., 2020).

Based on the characteristics of colorectal tumors, professionals have developed a classification system called consensus molecular subtype (CMS). These subtypes, while not specially used for this reason, can predict patient prognosis and outcome based on genetic profiles. As an attempt to better understand the pathways and biomarkers of CRC, the CMS groups (CMS1-CMS4) may pave the way for future treatments (Dienstmann, 2019). Although the paper has not discussed the respective CMS groups within each pathway, Figure 2 categorizes it.

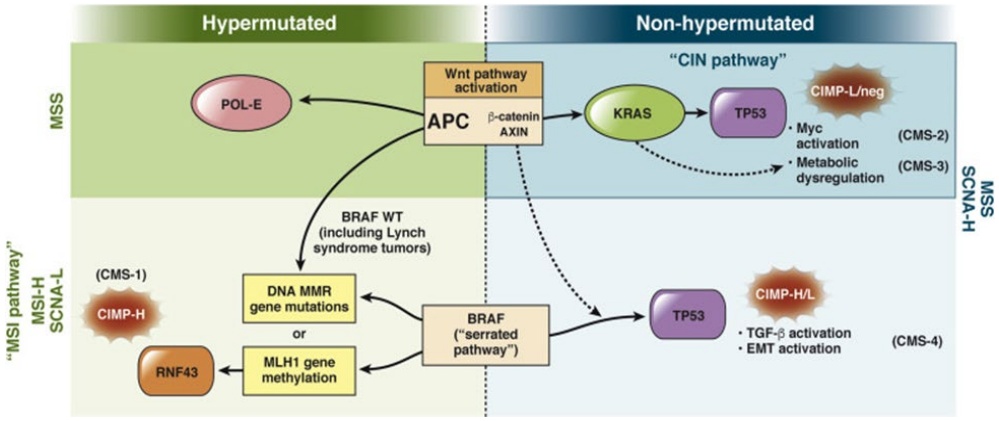

Figure 2. Pathways of Colorectal Carcinogenesis with CMS labeled. Source: Nguyen et al., 2020. Description: The figure illustrates the progression of colorectal tumors within various pathways. The top right box, labeled "CIN pathway", illustrates the major gene mutation progression that results in carcinoma. The bottom half of the table illustrates the two separate pathways of the serrated neoplasia pathway. The bottom left box, labeled "MSI pathway", illustrates the traditional MSI pathway; this pathway is the first serrated pathway. The bottom right illustrates the second serrated pathway.

Important Mutations, Their Physiologic Function, & Role in CRC Development

Discussing the various colorectal carcinogenesis pathways highlights the major gene mutations that occur in CRC progression. As Figure 2 portrays, mutations in the APC, KRAS, and TP53 genes and certain methylation biomarkers are key in the projection of CRC. Understanding each gene's function in CRC development, its mutational burden, and overall effect can help develop early detection tests. Because these mutations occur in tumor cells, the exfoliation of these cells occur faster in the stool than the blood during colorectal carcinogenesis (Han et al., 2019). Thus, using stool DNA to determine if these mutations exist may be the best way to determine these mutations. While there are other gene mutations involved in colorectal carcinogenesis, they will not be discussed because they are beyond the scope of this paper. Each of the gene's functions discussed in this section is shortened, and key details may be left out for the sake of simplicity.

The APC gene, classified as a tumor suppressor gene, regulates defective cells from transitioning between the G_1 and S phase of the cell cycle. The Wnt signaling pathway, as previously discussed, when activated helps native colonic and cancer cells survive in the colonic crypts. Normal levels of β-catenin control the migratory behavior of these cells by allowing them to shed between 3 to 7 days. If too much β-catenin accumulates, it may lead to the development of cancer cells staying in the colonic crypts. Eventually, this leads to the formation of polyps. Hence, the downregulation of β-catenin by APC is important in the colorectal carcinogenesis regulation. A study explored the possibility of testing for APC mutations from fecal DNA of various individuals using digital protein truncation. The study focused on the 1210 to 1581 codon length because most sporadic tumors showcased mutations in this region.

Fecal DNA from 74 patients (28 with Dukes' stage B2 colon cancer, 18 with adenomas, and 28 control) showcased 0.4 to 14.1 percent of APC gene mutations. All of the 28 control patients showed negative results. Positive results occurred in 17 of the 28 colon cancer patients and 9 of the 18 patients who had adenomas (Traverso et al., 2002).

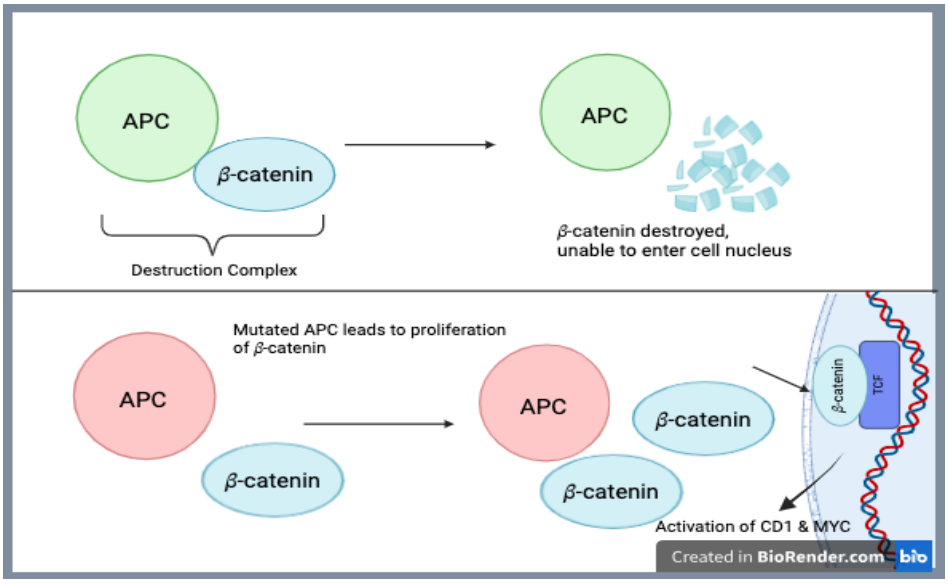

Figure 3. Simplified Schematic of the Effect of APC Gene Mutation on Cell Growth. Created using Biorender
Description: The top bow illustrates normal APC function, where it successfully degrades *β*-catenin by forming a destruction complex. The bottom row, however, illustrates APC's inability to form a destruction complex with *β*-catenin, thus allowing *β*-catenin to be activated and enter the cell's nucleus to induce transcription of genes related to CRC promotion.

The KRAS gene, part of the larger RAS gene family, is a tumor suppressor gene responsible for cell division, apoptosis, and cell cycle growth arrest by communicating and relaying signals to the cell nucleus from the outside. The KRAS protein is regulated between its active and inactive states. KRAS is activated when it binds to GTP, allowing KRAS to interact with other proteins that regulate the cell cycle (Divjak, 2020). When GTP transforms into GDP (which is governed by several intracellular signals including GAP), KRAS returns to its inactive phase. A single mutation within the KRAS protein can cause it to always remain active, leading to uncontrolled cell growth and division (Jančík et al., 2010). A study utilized droplet digital PCR (ddPCR) to determine mutations of the KRAS G12D gene from 70 CRC patients. KRAS oncogene mutations are usually found in codons 12 and 13, especially G12D (known to occur in 13%-14% of all CRC cases). DNA extraction from the 70 stool samples were taken. Of the 10 patients who were known to have a KRAS G12D mutation through pyrosequencing, 8 were found positive using ddPCR. Of the 8 patients, 6 were from early-stage tumors. Moreover, the KRAS G12D mutation was detected in 80% of all fecal DNA (Olmedillas-López et al., 2017).

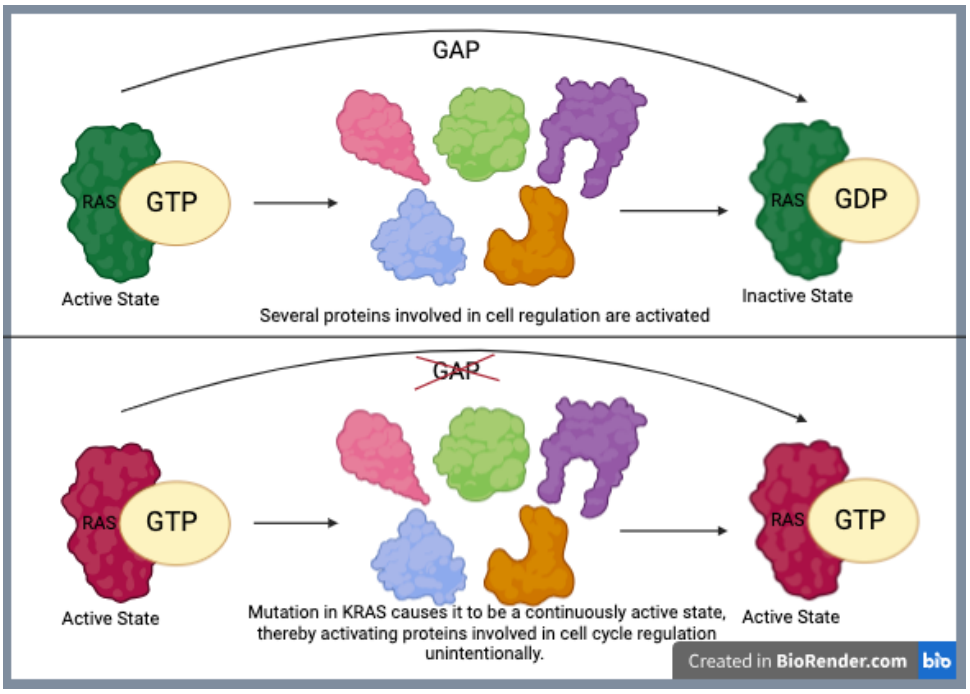

Figure 4. Simplified Schematic of the Effect of KRAS Gene Mutation on Proteins Involved in Cell Cycle Regulation. Created using Biorender. Description: The top bow illustrates normal KRAS function, in which it activates several proteins involved in cell cycle regulation; however, the bottom box illustrates KRAS' continuously active state, resulting in the upregulation of the cell cycle.

The TP53 gene is also referred to as the "guardian of the genome" because it plays a vital role as a tumor suppressor gene. p53 (the protein created by TP53) is responsible for DNA repair, apoptosis, and cell senescence (Liebl and Hofmann, 2021). MDM2, a protein that negatively regulates p53, increases p53 levels when DNA damage is found, preventing the cell from entering the S phase by increasing p21 levels thereby inhibiting CDK complexes. This increase in p53 initiates a long, complicated repair process of DNA or apoptosis if the damage is too great. When p53 is mutated, it can lead to its inactivity, allowing damaged cells to continue through the cell cycle and proliferate uncontrollably (Shankar, 2016). Mutations in TP53 are found in over 43% of CRC cancers (Liebl and Hofmann, 2021), and occur when adenomas transform into cancers (Armaghany et al., 2012). A study conducted to explore the possibility of testing for TP53 gene mutations in fecal DNA tested the tumors of 25 CRC patients, of which 11 (44%) had p53 gene mutations. Of the 11 patients with p53 mutations, PCR performed on exons 5-8 from fecal DNA had a positive result in 7 patients (64%). Additionally, 5 out of the 25 patients tested negative for FOBT. Three of the five patients had positive p53 mutation results, however. The results of this study showcase the ability to use p53 gene testing as an early screening method, especially for false-negative results from FOBT stool tests (Eguchi et al., 1996).

DNA methylation is an epigenetic silencing tool to turn genes "on" or "off". This process is extremely important to how cells function, including regulating a cell's growth, stability, and development. If an error in DNA methylation occurs, such as the deregulation of tumor suppressor genes, it can lead to cancer (Phillips, 2008). Because DNA methylation occurs early in carcinogenesis, it is a promising biomarker for early detection. In China, DNA methylation testing kits for early detection of 20 biomarkers are approved, half of which are used for CRC. However, testing multiple biomarkers can increase the sensitivity of early detection for CRC (Gao et al., 2024). A study was carried out that tested the presence of methylated SDC2 in patient stool. Single stool samples were collected from 585 individuals, which was tested for methylated SDC2 from PCR of isolated DNA. Of the 585 individuals, 245 had CRC. The study had an overall sensitivity and specificity of 90.2% in detecting CRC (221/245). Overall specificity for early

stages (0-II) was 89.5%. Specificities with respect to the clinical stage of CRC (0-IV) were 100% (3/3), 85.5% (47/55), 91.4% (64/70), 89.6% (86/96), and 100% (21/21), respectively (Han et al., 2019).

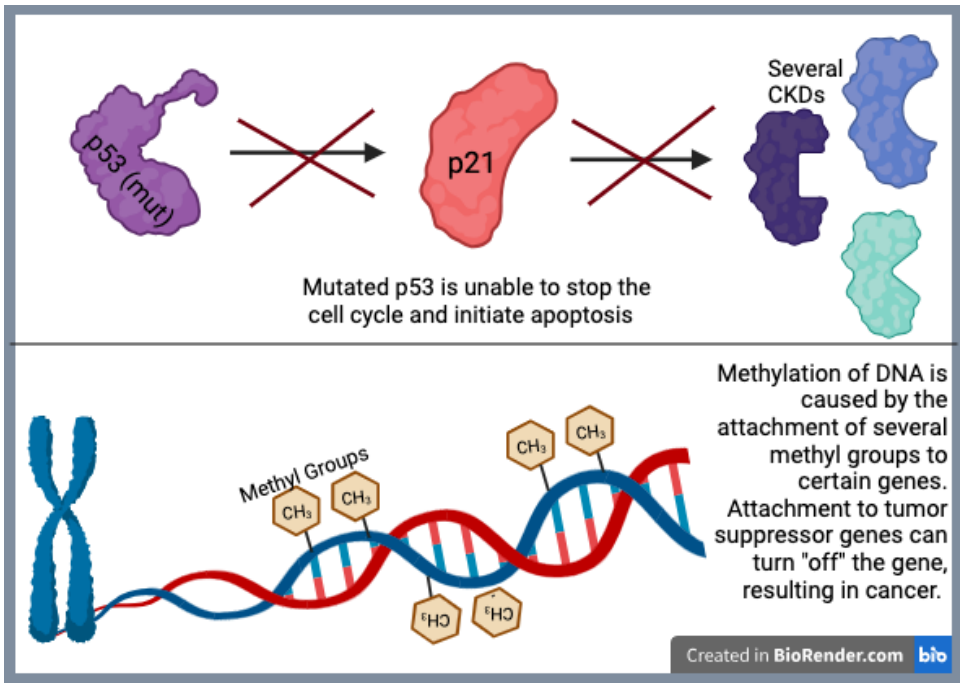

Figure 5. Simplified Schematic of the Effect of TP53 Gene Mutation on Proteins Involved in Cell Cycle Regulation & What Gene Methylation Is Created using Biorender. Description: The top box illustrates the effect of TP53 mutation on the cell. If there is DNA damage in the cell, but TP53 is mutated, it will not increase p21 levels and inhibit CDK binding, leading to uncontrolled cell division. The bottom box illustrates that DNA methylation, which is the attachment of the methyl (CH_3) group to the gene, results in its 'silencing'.

Combination Gene Biomarker Testing & Relevant Technology in CRC Early Detection

The previous section highlighted the major gene mutations and methylation markers that can lead to CRC. Although testing each mutation individually showed promising results, testing in combination may provide more efficient, sensitive, and reliable results. This section of the article will review studies that have been performed on a few of the said gene mutations in combination and the technology used, including PCR, quantitative PCR, and methylation-specific PCR (MSP), and next-generation sequencing, (NGS).

A recent study has explored the possibility of testing multiple gene mutations in combination by retrieving 45 preoperative and 41 postoperative stool samples and 54 tumor tissue samples from patients in Taizhou's People's Hospital. This included 20 preoperative and 20 postoperative control stool samples from those who had benign polyps or no CRC. Using NGS, the study identified APC (11.11%), KRAS (25.93%), and TP53 (37.04%) as the most significant mutations found in preoperative stool, compared with post-operative stool. Interestingly, the APC mutation frequency was relatively the same in preoperative and postoperative stool, which may have been attributed to a small sample size. With a 66.67% sensitivity and 68.97% negative predictive value of the KRAS-TP53 combination gene testing, the study concluded that this may be a promising method for early detection of CRC (He et al., 2022).

Another study used fecal DNA from 1142 patients (180 CRC, 60 advanced adenomas, 902 negative cases) to determine if combination methylation markers (SEPT9, SDC2, NDRG4, SFRP2, BMP3) can be used for early

detection. The study utilized bisulfate-conversion free DNA to reduce fragmentation, loss, and purification time. After extracting DNA, making bisulfite-conversion free, and putting it through PCR, the entire process took 6 hours, compared to the traditional 36 hours. The study found that testing SEPT9, SDC2, and SFRP2 in combination was promising in the 180 CRC patients, with 94.11% sensitivity and 89.21% specificity. Additionally, the sensitivity in detecting advanced adenomas was 38.33% (Liu et al., 2021).

A study utilizing combination gene mutation (KRAS/BRAF/APC) and methylation marker testing (SDC2/SFRP2) from fecal DNA of CRC, advanced adenomas (AA), non-advanced adenomas (NAA), and other gastrointestinal diseases patients achieved an 88.57% sensitivity with a 93.64% positive predictive value using the qPCR and MSP technique. The sensitivity of this combined testing panel increased as the CRC stage and lesion size of the adenoma increased. Additionally, this testing technique achieved a 79.49% specificity for the NAA group.
The test concludes that this testing panel may be an appropriate screening method for CRC (Lin et al., 2022).

Most studies, clinical trials, and tests utilize technology to determine gene mutations and specific methylation markers. These technologies include PCR, quantitative PCR, and methylation-specific PCR (MSP), and next-generation sequencing, (NGS). PCR allows DNA to be amplified, making it easier to detect minute changes in specific DNA sequences. qPCR, although very similar to PCR, utilizes a fluorescent dye and fluorometer, allowing PCR to be monitored in real-time (ThermoFisher Scientific, 2020). MSP utilizes two primers to detect methylated DNA. The first primer modifies DNA (using sodium bisulfite) by converting all unmethylated DNA bases to uracils, keeping methylated bases unchanged. The second primer amplifies methylated cytosines (Galm & Herman, 2005). NGS is a sequencing device that allows medical professionals to sequence DNA within several hours, at low cost. This device can locate mutations in the DNA, and holds an edge in personalized treatment, especially for cancer. Although the test utilizes complex techniques, including PCR, it is a vital tool in identifying genetic mutations (Guan et al., 2012).

Current Clinical Trials for CRC Early Detection

This section will focus primarily on a few current clinical trials for CRC early detection that explore the potential of various biomarkers and its efficiency in detecting CRC, as shown in Table 1.

Table 1. Current/Ongoing Clinical Studies on Novel Strategies for CRC Early Detection

Trial Code	Source of Biomaterial	Biomarker(s)	Study Status	Results
NCT00843375	Blood, Stool, & Tumor Tissue DNA	Methylated BCAT1/IKZF1/LINE1, galectin-3 ligand, TIMP1	Recruiting	N/A
NCT04739722	Stool DNA	Multi-target stool RNA test (Colosense) utilizing 8 biomarkers	Completed	94.4% detection of CRC, 46% of early lesions (Barnell et al., 2024).
NCT00855348	Blood DNA	SEPT9	Completed	Non-CRC cases: Sensitivity of 48.2% CRC cases (I-IV): 35.0%, 63.0%, 46.0% and 77.4%, respectively, specificity of 91.5% For Advanced Adenomas:11.2% sensitivity

NCT04823793	Stool DNA	Methylated ADHFE1/SDC2/PPP2R5C and FIT	Completed	Identified 75.0% of adenoma and 84.6% of CRC cases (Church et al., 2013).
NCT05779553	Blood DNA	TEM1	Not Yet Recruiting	N/A
NCT04722055	Stool DNA	Multi Gene Methylation Detection through Fluorescent PCR	Completed	Unknown
NCT02596113	Blood & Tumor Tissue DNA	hMLH1/K-Ras, B-Raf/ccfDNA	Completed	Unknown
NCT00507598	Blood, Urine, Stool & Tumor Tissue DNA	Metabolomic-Based Detection of CRC	Completed	Unknown
NCT01270360	Blood, Stool, & Tumor Tissue DNA	DNA methylation of Wif1/PENK/NPY	Completed	35% of asymptomatic patients and 50% of CRC patients showed a validation of cumulative methylation index ≥ 2 (Sobhani et al., 2019).
NCT03699163	Breath	Volatile organic compounds	Completed	Comparing CRC and non-CRC cases: 79% sensitivity, 86% specificity, and 97% negative predictive value (Vernia, 2021).

In addition to analyzing stool samples, which is the focus of this study, Table 1 includes other sample sources for testing, such as blood, tumor tissue samples (through a biopsy), and breath samples. There are currently numerous studies that utilize various biomarkers in CRC development, which are not just limited to genetic mutations or methylation. For example, NCT05779553 uses the overexpression of TEM1 (Tumor Endothelial Marker 1) in tumor vascular cells, which is often five, ten, or twenty-fold the quantity found in normal cells, to diagnose CRC. Another example is the use of volatile organic compounds (VOC) in patient breath (NCT03699163). VOCs found within the body are a product of microbial metabolism and the external environment. Most VOCs derived from microbial metabolism have recently been shown to be a marker for disease progression, especially CRC (Vernia, 2021). This biomarker is being developed as a part of the COBRA1 screening test for CRC utilizing multivariate discriminant analysis technology and its results show promise (Woodfield et al., 2022). NCT04739722 utilizes eight RNA markers (ACY1, AREG, CDH1, EGLN2, GAPDH, KRAS, SMAD4, & TNFRSF10B) known to be expressed in neoplastic development in the colon & FIT in a test known as a multi-target stool RNA test. The test utilizes digital droplet PCR and Bio-Rad (QXD) to determine if stool tests contained these biomarkers and the FIT test method. mt-sRNA has shown to be promising in CRC early detection, identifying 46% of lesions and 94% of CRC (Barnell et al., 2024). Lastly, NCT00507598 utilizes metabolomics, which are results of cellular metabolism that are known to be altered due to cancer. Although the results of this trial is unclear, based on previous trials by the same investigators, 158 metabolomics were monitored, distinguishing between CRC, healthy or polyp patients with a sensitivity of 0.96 and specificity of 0.89 (Zhu et al., 2014), identified several metabolomics that were altered in neoplastic tissue and blood DNA through nuclear magnetic resonance (Chen et al., 2015), and utilized five metabolomics (succinate, N2, N2-

dimethylguanosine, adenine, citraconic acid, and 1-methylguanosine) in CRC patients with a testing panel, resulting in 0.83 sensitivity and 0.94 specificity (Zhu et al., 2015).

The remaining trials use methylation markers. NCT00855348 utilizes methylation of SEPT9 from blood DNA to detect CRC. Using PCR and commercially available assays, it produced a standardized sensitivity of 48.2% (Church et al., 2013). NCT04823793 uses qMSP and PCR to test for combination gene methylations (ADHFE1/SDC2/PPP2R5C), in addition to FIT, to diagnose CRC. Interestingly, this study takes the racial background of a patient and found that specific gene methylation values varied, including that the SEPT9 and CRC relationship was stronger among Caucasian populations (Cheng et al., 2021). In addition to using a gene methylation marker panel, qMSP, and FIT, NCT01270360 experiments with fecal microbiota transfer from humans to rats to explore the effect of virulent microbiome gene markers (some include Firmicutes, Clostridia, and Clostridiales) on CRC development (Sobhani et al., 2019). Although NCT00843375 is underway, it also uses multiple methylation markers like the trials discussed previously. Results about NCT04722055 and NCT02596113 are also unclear. However, previous research conducted by the investigators of the respective trials have shown a variety of biomarker testing for CRC detection, such as KRAS/BRAF mutations, miRNA, mRNA, LncRNA markers, and endoscopy patient results.

Conclusion

To combat the growing trend of CRC death rates, it is vital that new, non-invasive testing methods are developed for regular use, increased patient adherence, and dependable results. Because cancer occurs as a result of an accumulation of several genetic mutations and methylations within the body, it is paramount to monitor such changes in genes to diagnose CRC earlier. Utilizing genetic biomarkers, including DNA mutations and methylation, is a promising method for CRC diagnosis.

This paper found that combination genetic biomarker testing for mutations in the APC, KRAS, and TP53 genes and certain methylation biomarkers (SDC2, SEPT9, etc.) using stool DNA is an encouraging testing method for CRC. These biomarkers were identified by examining the major gene players in the colorectal carcinogenesis pathway. While testing these genes individually has also shown promise and existing tests (such as Cologuard) use some of these markers, simultaneous testing of several under-searched biomarkers can reduce false-positive or false-negative readings, increasing accuracy and efficiency of CRC screening. Additionally, utilizing these biomarkers in screening methods should become more common in the future.

CRC can be exacerbated for several reasons, including an individual's lifestyle and family history. However, because of its insidious onset, detecting CRC early-on can be difficult. That is why it is crucial that all individuals, regardless of their symptoms, age, and family history, undergo regular screening. If an individual has unusual symptoms, such as blood in stool, they should make it a habit to reach out to their medical professional immediately and develop a self-driven approach in their health.

Limitations

This paper has a few limitations. First, the markers that were identified by examining the colorectal carcinogenesis pathway may have missed certain genes, such as BRAF. Secondly, using genetic mutation and methylation markers are not the only ways to detect CRC. For example, using the overexpression of certain proteins, virulent bacteria, and RNA markers can also be considered in the approach in detecting CRC. Third, this paper does not explore the implications of certain family history diseases/conditions, such as Lynch Disease or FAP, on the colorectal carcinogenesis pathway. Thus, certain gene expressions may be altered, and the precautions taken may vary among these individuals. Finally, the studies and clinical trials that have been explored are not exhaustive, so the likelihood of finding certain studies with differing outcomes is unlikely, but still probable. Some important publications may have also been

missed. Although this research is a secondary literature review, the ideas expressed in this paper were found through extensive research, cross-referencing several materials, and professor guidance.

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