

# Mutations by Next Generation Sequencing in Stool DNA from Colorectal Carcinoma Patients – A Literature Review and our Experience with this Methodology

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**Abstract:** It is well-known that colorectal carcinoma is a disease involving multistep carcinogenesis (hyperplasia-adenoma-carcinoma-metastasizing carcinoma). It is also a disease where therapeutically important driver mutations (especially in the EGFR signaling pathway) have been identified. Since genetic mutations can serve as good diagnostic and predictive markers, their reliable detection in the early stages of the disease and also in the follow-up of treatment efficacy is crucial. There is a fundamental problem encountered with the commonly used formalin-fixed paraffin-embedded (FFPE) specimens from biopsied tumor tissue i.e. it is unlikely that the material for the mutation analysis will be available in either the early stage of the disease or during the treatment period. Therefore recently attempts have been made to identify reliable markers from plasma/serum or from stool specimens. In particular, non-invasive stool specimens have been speculated to represent the situation of ongoing tumorigenesis and thus they can be used to assess treatment efficacy in the follow-up of the patient.

The key aims of this paper are firstly, to review the key methodological points when studying genomic alterations in DNA extracted from cells in stool specimens, and secondly, to review results related to biomarker screening and their therapeutic importance. A further aim is to present our new findings by focusing on the issues inherent in Next Generation Sequencing of stool specimens from patients with gastrointestinal tumors. Even though the focus of our paper is human genomic alterations in stool specimens, in our “future aspects” chapter, we also deal with the bacterial spectrum and its possible interaction with the genomic mutations.

**Keywords:** Mutation, DNA, Stool specimen, Colorectal carcinoma, Next generation sequencing.

## 1. INTRODUCTION

Colorectal carcinoma (CRC) is one of the major causes of cancer-related mortality and morbidity. It is a multistep process in which carcinogenesis progresses from the innocuous epithelial hyperplasia to terminal metastatic cancer. The etiological and pathological elements underlying CRC development are complex, complicated and heterogeneous. It is known that contributing factors include dietary and lifestyle factors, as well as the presence of different genetic mutations [1,2].

It is known that patients with CRC may display many mutated genes; these mutations can be in the oncogenes or tumor suppressor genes, and when these mutations provide a fitness advantage to the cell, they are called driver mutations. Recent sequencing-based studies have clarified many of the somatic mutations that are often associated with CRC, with the most frequent being in *APC*, *KRAS*, *PIK3CA*, *SMAD4*,

*BRAF* and *TP53*. In addition, many important pathways in the initiation process of CRC have been revealed e.g. the WNT, RAS-MAPK, PI3K, TGF $\beta$ , TP53 and DNA mismatch-repair pathways [3].

*APC* gene mutations play crucial roles in both familial adenomatous polyposis and sporadic CRC.  $\beta$ -catenin has been identified as a binding carrier for *APC* and it is a very important component of the Wntless/Wnt signal transduction pathway. In the presence of the Wnt signal in cancer cells,  $\beta$ -catenin binds to DNA in the nucleus where it acts as an essential co-activator of transcription. Furthermore, *APC* may control cell adhesion since  $\beta$ -catenin is an essential component of the adherence junctions, where it is the link between E-cadherin and  $\alpha$ -catenin, as well as between actin and actin-associated proteins. It is apparent that the *APC* gene encodes a multifunctional protein which participates in several important cellular processes such as cell adhesion, signal transduction and even in microtubule assembly [4].

*KRAS* gene mutations are present in approximately 40% of CRC cases [5]. The function of *KRAS* is to

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transmit extracellular signals from EGFR into intracellular cascades. *KRAS* mutations have an important predictive role in determining resistance to anti-EGFR therapy i.e. CRC patients with a *KRAS* mutation do not benefit from anti-EGFR targeted therapy [5]. *BRAF* acts as a downstream effector of *KRAS* and *BRAF* mutations have been reported to predict poor survival in CRC patients and they have also been related to anti-EGFR drug resistance [6,7]. *TP53* gene mutations are also some of the most frequently encountered genetic mutations in human cancers including CRC [3].

More than 50% of CRC patients are only diagnosed at advanced tumor stages and by that time there is a poor prognosis. It is unfortunate that the initial stages of CRC are usually asymptomatic, since early diagnosis has been associated with a better cure rate and prolonged survival [8]. At present, colonoscopy is the most sensitive method available for CRC diagnosis but it suffers from several limitations e.g. lack of patient compliance, risk of complications and the need of visible lesions to be detected. There is a fundamental problem with using formalin-fixed paraffin-embedded (FFPE) specimens for the mutation analysis since the biopsied tumor tissue is almost invariably taken from patients in advanced stages of the disease; it is unusual to acquire material from either the early stages of the disease or during the treatment period [9]. Therefore the need for a new sensitive method for early detection and screening has been one major scientific goal over the last fifty years [10]. Although currently established, fecal occult blood testing (FOBT) has also limitations because of its low sensitivity in detecting adenomas and early stage CRC (bleeding from tumor lesions in CRC usually appears in the late stages of the disease) and moreover, bleeding can occur in other conditions which are not cancerous [11,12].

## 2. STOOL BIOMARKERS IN CRC PATIENTS

The human stool is a mixture of undigested food residues, endogenous secretions, microflora, and exfoliated cells from colonic epithelium. Moreover, many other cellular types are exfoliated into the human stool. Inflammatory cells like leukocytes can be detected in stools from both healthy individuals and cancer patients. Blood cells may be present due to bleeding from colonic epithelium not only in CRC but also with other lesions [13].

Many different stool markers have been proposed to assist in the detection of colonic neoplasia. These

markers can be broadly classified into three main categories according to their mechanism of lumen entry, i.e. leaked, secreted, and exfoliated markers [13]. Leaked markers include hemoglobin which is the basis of the conventional FOBT [14]. Other leaked markers include calprotectin, a cytosolic leukocyte protein, the levels of which may be elevated in cases of adenoma and cancer and it can be used for CRC screening, achieving almost the same sensitivity as the FOBT [15]. The secreted markers include abnormal glycosylated mucins (mucus component) associated with colorectal neoplasia [16]. Exfoliated colonocytes and their constituents represent a diverse class of stool markers for CRC. The shedding of colonocytes from cancerous lesions differs from the process in normal colon in both qualitative and quantitative terms, with much greater shedding from colorectal tumors [17].

### Human and Bacterial DNA in Stool

Stool contains DNA from both exfoliated epithelial cells, blood cells and also from intestinal bacteria that may provide helpful information about the health status of the colon. The human DNA concentration in stool represents about 0.1% of the total DNA, with the remaining 99.9% being non-human, either from microflora or diet [18]. It has been demonstrated in several investigations that patients with CRC exhibit increased levels of human DNA in their stools [18]. The separation of bacterial DNA from its human counterpart in stool specimens has been a challenge for many years. For gene profiling on stool specimens, human specific probes or primers are generally used if we are interested in changes occurring in human cells, and on the other hand bacteria specific probes/primers are used if there is an interest in bacterial changes. Because culture conditions for human cells and bacteria are different, it is possible to get pure cell yields *in vitro* and thus DNA, too. These culture-based discrimination systems are not, however, generally used for gene profiling.

The DNA present in stools can be either cell-free or cellular. Several authors have reported the presence of cell-free DNA in stool specimens, plasma, urine, and various body fluids. In healthy individuals, the origin of this cell-free DNA is apoptosis of cells with the subsequent release of their degraded DNA [19]. In cancer patients, there are elevated levels of cell-free DNA in stool specimens, but the origin of this DNA is still debatable. Some authors have attributed this elevation to the abnormal apoptotic pathways in cancerous lesions, while others believe that it

originates from micro-metastatic tumor cells shed into the stool or the circulation [20,21].

A highly diverse microbiota lives in the colon; this is essential for the normal function of the intestine [22,23]. Recent studies have revealed that bacteria and their metabolites may have a role in CRC progression [24,25]. For example, acetaldehyde-producing bacteria and sulfate-reducing bacteria have been implicated as contributing factors to CRC due to the inflammatory and tumor-inducing effect of their metabolites [26,27]. However, other bacterial metabolites may reduce the risk of CRC development; including butyrate and other short chain fatty acids producers. These types of bacteria may be beneficial to human colon health [28].

### 3. STOOL DNA MARKERS

Fecal DNA from patients with CRC is divided into subsets of both non-apoptotic long DNA (L-DNA) and short DNA. L-DNA originates from cancerous or precancerous cells, which have been shed from dysplastic mucosa. Resistance to apoptosis is an epigenetic phenomenon that occurs in CRC, thus maintaining longer fragments of DNA (800 bp). In contrast, the apoptotic process in normal colonic mucosa cleaves DNA into 200 bp fragments resulting in the appearance of short DNA fragments [29]. Both the qualitative and quantitative analysis of stool DNA from patients with CRC have shown it to possess a higher integrity than stool DNA from healthy subjects [30]. Some authors reported a high frequency of L-DNA from distal CRC when compared with proximal CRC, thus suggesting that it may differ according to the tumor site and other clinicopathological parameters [30].

In August 2014, the FDA approved the multi-target test Cologuard as a non-invasive test for screening of CRC from stool DNA [31]. This kit searches for mutated human DNA and hemoglobin in stool; it does not require the usual preparation procedures for colonoscopy or some other screening tests, and can be used easily even at home. Since false positive results may occur, positive test results should be confirmed with colonoscopy. In October 2014, the Centers for Medicare & Medicaid Services (CMS) decided to recommend Cologuard for screening of adults with an average risk of CRC [31].

### 4. STOOL DNA ISOLATION

Stool DNA analysis is based on colonocyte exfoliation from tumor, and for this reason, the most

important factor is to gather a sufficient amount of DNA from colonocytes as there is always the possibility of collecting too low DNA amounts. In order to improve the sensitivity of detecting DNA mutations in stool, it is important to maximize the extraction and recovery of target fecal DNA. Handling of stool samples usually starts with collection before any surgical or medical interventions. For stool DNA analysis, the patient is instructed to give the sample in a tube container, and then the sample is either transported immediately or kept frozen and then transported frozen in an ice box container. Then storage at either -80°C [32–34] or at -20°C [35–38], although some studies have also reported storage in liquid nitrogen [39,40].

The literature describes a variety of different methods that have been successfully applied for DNA isolation. However, slightly different results can occur depending on whether the isolation methods are equally efficient at isolating small and large DNA fragments. The most commonly used methods are ready-to-use extraction kits like QIAamp DNA Stool mini kit (QIAGEN, Hilden, Germany) [33,40], or Nucleon PhytoPure DNA extraction kit (Amersham Life Science Inc., Arlington Heights, IL) [35], or phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) for extraction, and isopropyl alcohol for DNA precipitation [32], followed by purification of the DNA by streptavidin-coated magnetic beads [34,36].

### 5. STOOL DNA- BASED ANALYTIC METHODS

Most of the DNA analysis studies from stool specimens have focused on the detection of mutations or DNA methylation of specific genes, loss of heterozygosity (LOH) or microsatellite instability mostly by PCR and/or Sanger sequencing [41] or pyrosequencing [42]. The diagnostic biochip array has also been used in the detection of *KRAS* mutations in stool DNA [43].

In recent years, Next Generation Sequencing (NGS) has opened up new avenues for studying multiple gene mutations during a single experiment. Although this technique is now finding applications in cancer diagnostics, at present not many researchers have exploited NGS techniques for identifying genetic mutations in fecal DNA in CRC.

Recently, Russo *et al.* [44] described the feasibility and high sensitivity of single molecule third generation sequencing for the detection of CRC mutations in stool DNA. They applied single molecule real time circular

consensus sequencing (SMRT-CCS) to detect *APC* gene mutations at a very low frequency of 0.5% without encountering any false positives.

In our research group, we have utilized amplicon-based NGS techniques to study mutations in important cancer genes from fecal DNA using the Ion Torrent PGM (Life Technologies, Carlsbad, CA). Previous studies have shown that Ion AmpliSeq panels possess high sensitivity at detecting mutations in FFPE samples from CRC patients using Ion Torrent technology [45, 46]. We have successfully used this method on stool DNA from CRC patients. Amplicon based NGS technology has a major advantage since it overcomes the problems encountered during stool DNA extraction due to the low amount of DNA required (as little as 10 ng).

The procedure used in our lab has been described in detail elsewhere [47]. In brief, the protocol is as follows; the first step is stool collection prior to surgical or medical procedures, in special tubes provided by the manufacturer (STRATEC Biomedical AG, Germany), and then immediate freezing to -20°C. For the DNA isolation, we have used both QIAamp DNA Stool mini kit (QIAGEN, Hilden, Germany) and the PSP Spin Stool DNA Plus Kit (STRATEC Biomedical AG, Germany), following the manufacturer's instructions. The DNA concentration is assessed using Qubit® 2.0 Fluorometer (Life Technologies) and the Qubit® dsDNA BR Assay kit. The extracted DNA is stored at -20°C.

For gene mutation analysis, we have used two different AmpliSeq panels:

- a) Ion AmpliSeq Colon and Lung Cancer panel, consisting of a primer pool of 90 amplicons from hotspot regions of 22 genes frequently mutated in CRC and lung cancer.
- b) Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) consisting of a primer pool of 207 amplicons from 50 genes.

Screening of CRC mutations by using the Ion AmpliSeq Colon and Lung Cancer Panel for tumor samples has been successfully applied and validated by eight cancer research groups from different European institutions; moreover, its accuracy has been confirmed recently in CRC patients [46]. One of the unique advantages of NGS technology is its ability to detect a wide range of multiple mutations within a single test run.

## Bacterial Biodiversity Analysis from Stool DNA

During the last few years, with the development of NGS technologies, it is now possible to determine the biodiversity of the human gut microbiota. The analysis of the bacterial 16S rRNA gene sequences in stool samples is now a generally accepted approach for studying and understanding the nature of the communities of fecal microbiota. Recently, an optimized protocol was designed to evaluate the fecal microbiota based on Ion Torrent PGM technology [48].

## 6. DIAGNOSTIC APPLICATIONS OF STOOL DNA ASSAYS

Over the last twenty years, many studies have been conducted to detect mutations in stool specimens as a non-invasive method to aid in the diagnosis of CRC patients (Table 1). In one of the first studies examining this topic that have been attempted in 2000, Ahlquist *et al.* [49] investigated stool DNA mutations for *KRAS*, *TP53*, *APC*, along with other markers (BAT26, and L-DNA) in both cancer specimens and in large adenomas. The authors reported a sensitivity of 91% for CRC and 82% for large adenomas by applying a multi-target DNA assay panel by PCR amplification. Koshiji *et al.* [50] investigated LOH at *APC*, *TP53*, and D9S162 in genomic DNA extracted from tissue, stool, blood and oral rinse in CRC and hereditary non-polyposis colon cancer patients. They concluded that LOH determined from the oral rinse and the stool samples matched those determined from the blood and the neoplastic tissue. Years later in 2003, another multi-target DNA assay panel resulted in a sensitivity of 63.5% for CRC and 43.7% for advanced adenomas [51]. Around the same time, a new innovative technique was introduced by Prix *et al.* [43] utilizing the diagnostic *KRAS* biochip for rapid detection of *KRAS* mutations in stool samples. All mutations were confirmed by sequencing and the success rate was 50% for early detection of CRC patients.

One year later, Calistri *et al.* [52] used an approach based on the analysis of fecal DNA for *KRAS*, *APC*, *TP53* mutations and microsatellite instability, detecting *KRAS* mutations in 11%, followed by *TP53* (6%), and *APC* (2%). In 2004, one large scale study compared fecal DNA analysis with a panel consisting of 21 mutations and other markers (including *KRAS*, *APC*, *TP53*, BAT26, and L-DNA) against the FOBT in screening for CRC. The resulting sensitivity was 51.6 % for the fecal DNA panel versus 12.9 % for FOBT [53]. Furthermore in 2005, Kutzner *et al.* [54] compared

Table 1: Studies Performed on Stool DNA from Colorectal Cancer Patients

Reference	Number of individuals				Detection Method	Targeted Genes			Remarks
	Control	CRC	Stage <sup>1</sup>	Adenoma (advanced)			Sensitivity	Specificity	
Ahlquist <i>et al.</i> [49], 2000	28	22	A-D	11 (11)	PCR	<i>KRAS</i> , <i>TP53</i> , <i>APC</i> , BAT-26, L-DNA	91% CRC, 82% adenomas	93 %	
Koshiji <i>et al.</i> [50], 2002	15	30	NR	0	PCR	<i>APC</i> , <i>TP53</i> , D9S162	81% CRC	86.70 %	
Calistri <i>et al.</i> [52], 2003	38	53	A-D	0	DGGE/SSCP	L-DNA, <i>KRAS</i> , <i>TP53</i> , MSI, <i>APC</i>	NR	NR	All mutations were confirmed by sequencing
Tagore <i>et al.</i> [51], 2003	212	52	NR	28 (28)	PCR	<i>KRAS</i> , <i>APC</i> , <i>TP53</i> , DIA, BAT-26	63.5% CRC, 43.7% advanced lesions	96 %	
Imperiale <i>et al.</i> [53], 2004	1423	31	NR	1051 (403)	PCR	<i>TP53</i> , <i>KRAS</i> , <i>APC</i> , BAT26, L-DNA	51.6% CRC, 18.2% high grade dysplasias	94 %	
Kutzner <i>et al.</i> [54], 2005	44	57	NR	NR	PCR	<i>APC</i> , BAT26, L-DNA	65 %	NR	
Matsushita <i>et al.</i> [55], 2005	116	83	A-D	NR	Direct sequencing	<i>APC</i> , <i>KRAS</i> , <i>TP53</i> , BAT26	71% CRC	NR	
Jin <i>et al.</i> [56], 2006	36	NR	NR	25 (20)	PCR, RFLP and direct sequencing	<i>KRAS</i> , BAT26, <i>BRAF</i>	80% adenomas and 100% HPs	92% adenoma and 100% HPs.	
Ahlquist <i>et al.</i> [57], 2008	75	19	NR	123 (123)	PCR	<i>KRAS</i> , <i>APC</i> , <i>TP53</i> , BAT26, L-DNA, vimentin	58% SDT-2 & 25% SDT-1	NR	
Ahlquist <i>et al.</i> [38], 2012	293	252	NR	227	QuARTS	<i>KRAS</i> , <i>NDRG4</i> , <i>BMP3</i> , vimentin,	85% CRC and 54% large adenomas	90 %	
Imperiale <i>et al.</i> [58], 2014	4457	65	NR	757	Quantitative Molecular Assays	<i>KRAS</i> mutations, aberrant <i>NDRG4</i> , and <i>BMP3</i> methylation	92.3% CRC, 42.4% advanced precancerous lesions, 69.2% high grade dysplasias	86 %	
Prix <i>et al.</i> [43], 2002	0	26	B-D	0	Biochip array, PCR, sequencing	<i>KRAS</i>	NR	NR	Success rate 50 %
Russo <i>et al.</i> [44], 2015	0	2	NR	0	SMRT-CCS, Illumina MiSeq, IonTorrent PGM	<i>APC</i> , <i>KRAS</i> , <i>TP53</i>	100% for SMRT-CCS	100% for SMRT-CCS	Comparison of 3 sequencing instruments
Armengol <i>et al.</i> [47], 2016	0	65	NR	0	IonTorrent PGM	Ion Ampliseq Colon and Lung Cancer panel	75%	NR	Success rate 80 %

1, Stage according to Dukes' classification; CRC, Colorectal cancer; L-DNA, Long-DNA; NR, Not reported; DGGE, denaturing gradient gel electrophoresis; SSCP, single strand conformation polymorphism; HPs, Hyperplastic polyps; SDT-1, stool DNA test-1 (21 tumor-specific point mutations); SDT2, stool DNA test-2 (*KRAS*, *APC* mutator cluster regions and methylation of the vimentin gene); QuARTS, quantitative allele-specific real-time target and signal amplification; SMRT-CCS, single molecule real time circular consensus sequencing.

the sensitivity and the specificity of FOBT and molecular analysis of fecal DNA for detecting alterations in three different markers *APC*, *BAT26* and *L-DNA*, obtaining overall sensitivities of 51% and 65% respectively. However, the combined application of both methods resulted in a sensitivity of 93% and an overall specificity of 89%.

In the same year, Matsushita *et al.* [55] successfully applied a new method for isolating colonocytes from naturally evacuated feces, followed by DNA analysis and cytology. The extracted DNA was examined for mutations in the *APC*, *KRAS*, and *TP53* genes using direct sequencing analysis with a reported sensitivity of 71% for CRC. Additionally, the stool mutation pattern was rather similar to the mutations present in tumor tissue. Another approach in 2006 exploited by Jin *et al.* [56] attempted to detect *BRAF*, and *KRAS* mutations, and *BAT26* microsatellite instability as stool-based molecular markers for the detection of colorectal adenomas and hyper-plastic polyps.

In 2008, a cross-sectional study conducted by Ahlquist *et al.* [57] on 4482 average-risk adults compared two different stool DNA tests (SDT): SDT-1 including 21 tumor-specific point mutations, and SDT-2 consisting of three tumor-specific markers (*KRAS*, *APC* mutator cluster regions and methylation of the vimentin gene). The study revealed that the latter assay could successfully detect three times more mutations in colorectal adenoma than the former assay, i.e. sensitivities of 58% and 25% for SDT-2 and SDT-1, respectively. Another case control study conducted by Ahlquist *et al.* [38] used archived stool samples from CRC, adenomas larger than 1 cm, and healthy controls to evaluate a mutant form of *KRAS*, four methylated genes and the  $\alpha$ -actin gene. This stool DNA test achieved a high rate of detection for all non-metastatic stages of CRC (aggregate 87% detection rate for CRC stages I–III). Furthermore, detection rates increased with higher adenoma size. A similar cross-sectional study by Imperiale *et al.* [58] targeted asymptomatic subjects between 50 and 80 years (considered to be at average risk). They investigated a stool DNA test which included quantitative molecular assays for *KRAS* mutations, aberrant *NDRG4* and *BMP3* methylation, and  $\beta$ -actin, plus a hemoglobin immunoassay and reported a detection sensitivity of 92.3% for CRC, 42.4% for advanced precancerous lesions and 69.2% for dysplastic polyps.

An alternative approach was recently described by Russo *et al.* [44]; they applied single molecule third

generation sequencing for detection of *APC* gene mutations in DNA from paired stool and tissue samples and claimed that it was possible to detect mutations at very low levels (0.5%).

In our experiments, the NGS assay was successfully applied on DNA from stool samples of CRC patients with a success rate of 80%, testing 22 genes commonly mutated in colon cancer [47]. The results of gene mutation analysis revealed both hotspot and novel mutations with subsequent confirmation of some mutations by PCR and Sanger sequencing. Paired tissue samples were analyzed by NGS, identifying the same mutations in both stool and tumor specimens, reinforcing the concept that DNA mutations in stool specimens represent mutations from tumor cells. The most frequently mutated genes were *TP53*, *KRAS*, *FBXW7*, *EGFR*, and *SMAD4*. However, the AmpliSeq Cancer Panel used in our protocol does not include the *APC* gene or the *ATM* gene, and therefore mutations in these genes, which are frequently present in CRC, could not be analyzed. One of the interesting findings was that alterations in the combined PI3K/MAPK pathway could be detected in about 70% of samples. The use of NGS technology made it possible to identify several mutations that were novel and not reported previously. This agrees with the outcomes of other studies, e.g. Seshagiri *et al.* [59] performed an exome sequencing analysis on 72 CRC samples and found that 98% of the detected mutations were novel. This indicates that NGS approaches applied to multiple driver genes are more sensitive than current conventional analytical techniques.

## 7. FUTURE PROSPECTS

Stool DNA analysis is a promising approach for investigating CRC since it is simple, non-invasive and has better patient compliance than colonoscopy. However, many challenging problems can be encountered along the way, starting from proper DNA isolation and ending with the need to detect genetic mutations at high sensitivity. The exploitation of amplicon-based NGS technology can overcome many of these problems due to the low amount of DNA required. Although NGS is applied in CRC diagnostics, evaluation of its high sensitivity in stool based DNA diagnostics still needs more studies. Stool DNA analysis by NGS technology might have therapeutic implications based on the genetic mutations detected. It is known that mutations in *KRAS*, *BRAF*, and *NRAS* mutations counteract the benefits of anti-EGFR treatment, and testing of these mutations is currently

recommended before initiating EGFR blockade therapy in metastatic CRC patients [60]. Therefore, the identification of these mutations may be helpful in guiding therapeutic decisions.

Additionally, NGS can be applied to investigate the fecal microbiota and can assist in understanding the bacterial alterations that might contribute to the development of CRC. Moreover, stool DNA analysis by NGS could be used to monitor disease progression or to predict relapse in pre-symptomatic individuals during follow-up.

In conclusion, fecal DNA analysis conducted by NGS techniques represents a promising tool for identifying CRC genetic mutations and seems likely that it will prove useful in the early diagnosis, follow-up and targeted therapy of this life-threatening disease.

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