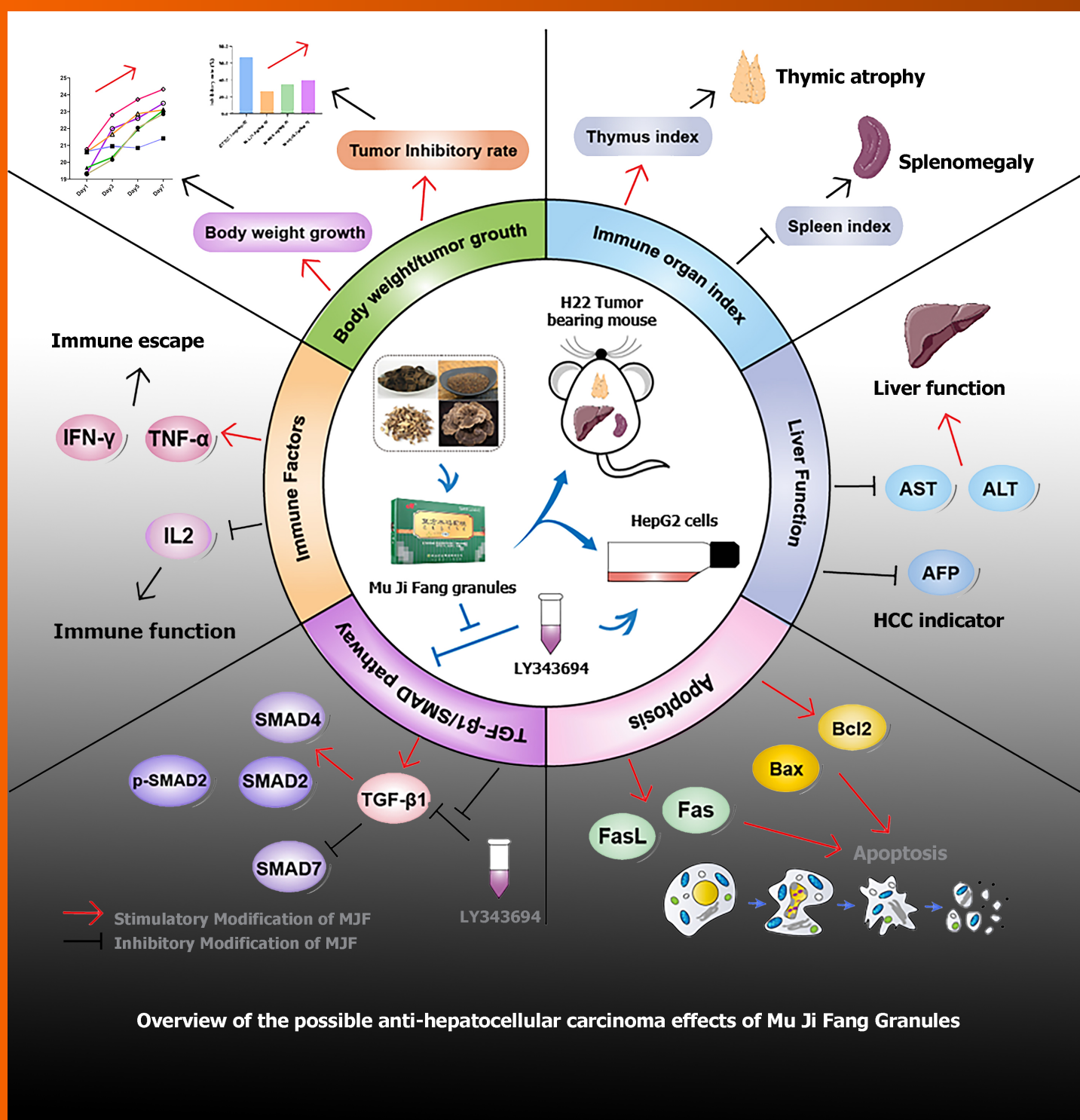


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WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, etc.

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Molecular methods for colorectal cancer screening: Progress with next-generation sequencing evolution

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Abstract

Currently, colorectal cancer (CRC) represents the third most common malignancy and the second most deadly cancer worldwide, with a higher incidence in developed countries. Like other solid tumors, CRC is a heterogeneous genomic disease in which various alterations, such as point mutations, genomic rearrangements, gene fusions or chromosomal copy number alterations, can contribute to the disease development. However, because of its orderly natural history, easily accessible onset location and high lifetime incidence, CRC is ideally suited for preventive intervention, but the many screening efforts of the last decades have been compromised by performance limitations and low penetrance of the standard screening tools. The advent of next-generation sequencing (NGS) has both facilitated the identification of previously unrecognized CRC features such as its relationship with gut microbial pathogens and revolutionized the speed and throughput of cataloguing CRC-related genomic alterations. Hence, in this review, we summarized the several diagnostic tools used for CRC screening in the past and the present, focusing on recent NGS approaches and their revolutionary role in the identification of novel genomic CRC characteristics, the advancement of understanding the CRC carcinogenesis and the screening of clinically actionable targets for personalized medicine.

Key Words: Colorectal cancer; Gut microbiota; Colorectal cancer screening; Next-generation sequencing

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Core Tip: Due to the multitude of host and microbial genetic factors, the optimization of colorectal cancer (CRC) biomarkers remains difficult. The advent of next-generation sequencing (NGS) methods has facilitated the identification of previously unrecognized CRC-related genomic alterations and the CRC relationship with gut microbial composition. Hence, we have summarized the diagnostic tools used for CRC screening in the past and the present, focusing on the revolutionary role of NGS approaches in the identification of novel genomic CRC characteristics, the advancement of understanding the CRC carcinogenesis and the screening of clinically actionable targets for personalized medicine.

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INTRODUCTION

Currently, colorectal cancer (CRC) represents the third leading cause of cancer-related deaths in men and women worldwide, and the American Cancer Society estimates that the number of new colon and rectum cancer cases in the United States in 2022 will be around 106180 and 44850, respectively[1]. Despite the great progress of modern medicine, such as the development of novel therapeutic methods and the advent of new high throughput sequencing technologies, the mortality of CRC patients remains relatively high due to the lack of specific biomarkers and therapies.

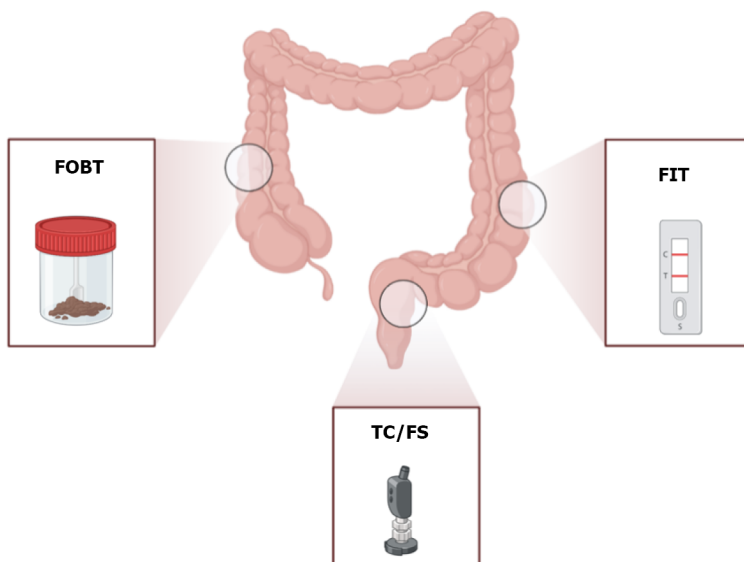
Nowadays, CRC incidence largely varies across the world, and it appears to be positively correlated with the Human Development Index. For instance, in 2020 Norway, the Netherlands and Denmark reported the highest age-standardized incidence rates (41.9, 41.0 and 40.9 cases per 100000 persons, respectively) while Guinea, Gambia and Burkina Faso showed the lowest age-standardized incidence rates (3.3, 3.7 and 3.8 cases per 100000 persons, respectively)[2]. Usually, these variations reflect differences in the availability of screening services and other factors such as geographic location, environmental factors (*e.g.*, polluted surface water sources), economic status and dietary and lifestyle habits[3].

At present, considering the difficulties in implementing significant lifestyle changes or common primary prevention strategies, screening and early detection represent the most powerful public health tool to reduce CRC mortality[4]. In general, an acceptable screening marker can only be considered by the health community if it respects specific parameters such as simplicity, safety and accuracy and has a known and defined suitable cutoff level[5]. Colonoscopy is considered the gold standard test for detecting CRC and promoting effectiveness in reducing its incidence and mortality. However, its high cost, invasiveness and reduced availability of necessary equipment hinder the establishment of organized screening settings, especially in poor countries[6].

In recent years, massive efforts have focused on next-generation sequencing (NGS) approaches to identify genes and microorganisms that are significantly associated with the malignancy due to the emerging evidence that intestinal microbial dysbiosis constitutes a crucial environmental factor in CRC onset and development[7]. Moreover, metagenomics approaches, considered a real revolution in the screening and diagnosis of different cancers, are also useful for the identification of novel potential markers for CRC diagnosis[8]. Hence, in this review, we summarized the diagnostic tools used for CRC screening in the past and the present, focusing on recent NGS approaches.

FECAL OCCULT BLOOD TEST

Since the 1970s, stool-based CRC screening was considered a successful non-invasive method with proven effectiveness given by the detection of high-risk polyps and early-stage malignancies that dramatically reduced CRC incidence and death[9] (Figure 1). The fecal occult blood test (FOBT) currently represents the early analysis for CRC screening that is recommended by the National Screening Committee[10]. This method is based on the detection of occult blood by measuring the non-protein portion of hemoglobin, the heme group, present in the stool. In particular, the heme present in a stool sample reacts with hydrogen peroxide-based developer to oxidize guaiac-infused paper, resulting



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Figure 1 Stool-based and visual colorectal cancer screening methods. FIT: Fecal immunochemical test; FOBT: Fecal occult blood test; FS: Flexible sigmoidoscopy; TC: Total colonoscopy.

in a blue color[4]. In general, FOBT has been shown to reduce both the incidence and the risk of CRC death with the advantages of ease of use and cheaper than other alternative screening approaches[11] (Table 1). Despite this, the FOBT method presents some limitations, such as the low sensitivity for colorectal adenomas that may not bleed or the specificity of the method that can be influenced by diet or drugs[12]; hence, Young *et al*[13] affirmed that FOBT is only suitable for limited colonoscopy resources with a need to constrain the test positivity rate[13].

FECAL IMMUNOCHEMICAL TEST

Different immunoassay methods have been used to measure the development of antibody-globin complexes, including immunochromatography, immunoturbidimetry and enzyme-linked immunosorbent assay (Table 1)[14]. For instance, the fecal immunochemical test (FIT) is used for the detection of microscopic amounts of blood present in the stool during defecation *via* the utilization of antibodies targeted to globin molecules (Figure 1). The antibodies preferably target lower gastrointestinal bleeding, making FIT easy to use, sensitive to low concentrations of globin and sufficiently flexible to adjust the cutoff concentration for positivity (the cutoff is usually selected with a risk threshold that would produce a specificity of 96.9% in the study group, matching the specificity of FIT at a cutoff of 20 µg Hb/g feces)[13,15]. Imperiale *et al*[16] tested individuals at average risk for CRC having an age comprised between 50 years and 84 years and documented that FIT detected 48 out of 65 colon cancers, showing a sensitivity of 73.8% and specificity of 96.0%[16]. The same specificity was observed among participants with negative results on colonoscopy, suggesting that FIT had fewer false positive results compared to stool DNA testing[17]. On the other hand, weaknesses of FIT tests are the low clinical sensitivity for both cancers (73%, 80%, 82% and 79% for CRC stages I, II, III and IV, respectively) and advanced adenomas (16%-34%) when used at a low cutoff and the limited detection of upper gastrointestinal bleeds because the hemoglobin undergoes degradation by digestive enzymes with a consequent reduction of the binding to FIT antibodies[18].

FLEXIBLE SIGMOIDOSCOPY AND TOTAL COLONOSCOPY

Randomized controlled trials showed that the visual inspection of colic mucosa through flexible sigmoidoscopy (FS) decreased CRC mortality and incidence by 22%-31% and 18%-23%, respectively (Figure 1)[19]. Overall, FS represents a safe test, but its use is limited to the distal colon and a combined strategy using FS and FOBT/FIT only increases the endoscopic workload and reduces patient participation without solving the problem. Instead, total colonoscopy allows direct visualization and polyp removal over the whole colon (Figure 1), has a very high sensitivity and specificity for CRC and is usually used as confirmatory for all other screening strategies (Table 1)[20]. Although total colonoscopy can lead to a decrease in CRC incidence (66%-90%) and mortality (31%-65%), many features (*e.g.*,

Table 1 Summary of the main diagnostic approaches for colorectal cancer screening

Technology	Approach	Sample types	Targeted and colorectal marker	Sensitivity/specificity for CRC	Advantages	Disadvantages
Chemical and immuno-chromatographic test	FOBT	Stool	Heme of hemoglobin	4%-25% / 95%	(1) Non-invasive; (2) Reduction of mortality (asymptomatic patients); (3) Colorimetric indicator; (4) Rapid and easy-to-carry out (self-testing); and (5) Commercially available test	(1) Low sensitivity for non-bleeding adenoma and advanced adenoma; (2) Specificity influenced by diet or drugs; (3) Must be done annually; (4) Risk of false positive results; (5) Three consecutive samples needed; (6) Only detects the blood present in the external layer of the stool; and (7) Confusing interpretation of the test results
	FIT	Stool	Globin molecules	62.0%-100% / 94.9%	(1) Easy to use; (2) Flexible cutoff concentration; (3) Sensitive to low concentrations of globin; (4) Single sample needed; (5) Combined with FOBT inferred mortality; and (6) No dietary restriction	(1) Insensitive to digested hemoglobin; (2) Poor sensitivity for advanced adenoma; (3) Sensitivity based on threshold value of hemoglobin; and (4) Detect more distal neoplasms
Visual inspection	FS	Distal colon	Polyps	100% / 100%	(1) Reduce colorectal cancer mortality and incidence; and (2) High susceptibility to detect adenomas	(1) Invasive process; (2) Not suitable for diabetic or psychotropic patients; (3) Expensive; (4) Serious harms for colonoscopy that increase with age; (5) Sigmoidoscopy was not effective for female screening (high risk for proximal colorectal cancer); and (6) Moderate-to-severe pain was reported for patients (bleeding, anxiety, <i>etc</i>)
	TC	Entire colon				
Sanger sequencing methodology	Single gene sequencing	Tissue; liquid biopsy	A specific gene in human tumor DNA cells	High sensitivity (input of DNA mutated quantity < 1%)	(1) Non-invasive (blood/liquid biopsy); (2) Some mutations were prominent in colorectal cancer; (3) Bioinformatic analysis not required; (4) Simple and less time consuming; and (5) No specialized instrument in laboratory	(1) Requires high-quality DNA; (2) Heterogenous mutations genes; (3) Risk of contamination with normal tissue; and (4) Low coverage sequencing
	ddPCR	Liquid biopsy. Tissue	Short amplicon sizes (< 100 bp) of human DNA	Very high sensitivity (input of mutated DNA quantity < 0.1% even with degraded DNA)	(1) Monitoring tumor burden in response to treatment and indicator of disease progression; (2) Precise measurement of copy number of mutated DNA and lower probability error (without standard samples); (3) Minimally invasive process; (4) Detects specific mutations; (5) Independent prognostic factor; and (6) Large target mutation	(1) No ability to detect benign lesions from plasma due to insufficient tumor burden; (2) Need an expensive instrument; (3) Limited prime-probe sets for each single nucleotide change; (4) No information in tumor-associated protein profiling; (5) Possibility of contamination with normal tissue; (6) Not strictly tumor specific; and (7) Necessity of cell search system
	MT-sDNA	Stool	Specific genes in human tumor DNA cells	66%-94% / 90%-96%	(1) Non-invasive test; (2) Acceptable cost; (3) Potential credibility; (4) No dietary restrictions (including food and medications); and (5) Widespread accessibility and multiple commercialized prototypes	(1) Lack of standardization or optimization of fecal DNA panels for high sensitivity and specificity; (2) Risk of contamination by microbial DNA; (3) No defined optimal interval for screening individuals; (4) Poor sensitivity for advanced adenoma; and (5) Must be repeated every 3 years
	Idylla system	Tissue; liquid biopsy	Specific genes in human tumor DNA cells	High sensitivity (input of DNA mutated quantity < 1%)	(1) Fully automated; (2) Real-time based-PCR molecular diagnosis system; (3) Without pre-analytical DNA extraction; (4) Lower cost and time requested for results; (5) Easily	(1) No detection of complex genomic variants; (2) Unknown mutations were not detected; (3) Cannot detect rare and complex genomic variants not included in the reference range; and (4) Less suitable when new

	Custom panel sequencing	Tissue; liquid biopsy	Specific genes in human tumor DNA cells	95%-100%/99%-100%	implemented in routine laboratory workflow; (6) Wide range of CRC-related mutations; and (7) Very sensitive to detect the most common CRC mutation	gene mutations appear
					(1) Decreased sequence cost; (2) Greater sequencing depth; (3) Simple and less time consuming; (4) Robust and tissue efficient; (5) Massive parallel multigene sequencing; and (6) Provide additional information (TMB levels/relevant mutated genes/heredity cancer genes)	(1) Low coverage sequencing; (2) There is no standardized procedure; and (3) Relatively long turnaround time of 3 d
Next generation sequencing	WGS/WES	Tissue; liquid biopsy	All exome and all genome in human tumor DNA cells	95%-100%/99%-100%	(1) Detection of large-scale mutations; (2) High coverage sequencing; (3) Complete definition of the genomic landscape for WGS; and (4) Complete mutation analysis panel without the repeated testing cost and reuse of material	(1) Require bioinformatics specialists; (2) Expensive; (3) Require good quality DNA; and (4) Relatively long turnaround time of 3 d
	Third generation sequencing	Tissue/stool/liquid biopsy	(1) Specific genes/WES/WGS in human tumor DNA cells; and (2) Microbes in stool	95%-100%/99%-100%	(1) Identification of large-scale rearrangement; (2) Sequencing errors do not release rearrangement; (3) High coverage sequencing; and (4) Fast and real time molecular diagnosis system	(1) High percentage of somatic errors; (2) Require bioinformatic specialists for assembling and analysis in laboratory; (3) Need specialized equipment in laboratory; and (4) Cannot detect some somatic mutations
	Metagenomic analysis	Stool/tissue	Microbial DNA in stool by Shotgun (all the DNA) or metabarcoding DNA (16S, ITS1, ITS2, 18S, <i>etc</i>)		(1) Microbial flora was more abundant than human cells in stool; (2) Benign lesions do not release human cells in stool; (3) Noninvasive diagnostic test; (4) Microbiota seems to play a role major in initiation and progression of CRC; (5) Test can be potentially used on all pathogen groups; and (6) Microbiota dysbiosis induces methylation of host genes	(1) Complex bioinformatics analysis; (2) Expensive; (3) Microbiota composition depends on sample preparation, conservation, extraction protocol and many other factors; (4) Need a healthy control group; (5) Many microorganisms (virus, bacteria, fungi) have not been identified and sequenced; (6) Metabarcoding analysis provides only taxonomic affiliation based in small region; and (7) Analysis results depends on reference database

CRC: Colorectal cancer; ddPCR: Droplet digital polymerase chain reaction; FIT: Fecal immunochemical test; FOBT: Fecal occult blood test; FS: Flexible sigmoidoscopy; MT-sDNA: Multi-target stool DNA; TC: Total colonoscopy; TMB: Tumor mutational burden; WES: Whole exome sequencing; WGS: Whole genome sequencing.

invasive, expensive and painful) dramatically reduce its acceptability as a first-line screening test; moreover, proper training programs for endoscopists as well as continuous quality assurance are necessary[21].

METHODS-BASED ON SANGER DNA SEQUENCING

It is currently well established that CRC development relies upon a stepwise acquisition of several chromosome mutations. The model of the adenoma-carcinoma progression, based on the accumulation of multiple mutations and epigenetic alterations, has been widely accepted[22]. Overall, there are two types of mutational events in sporadic CRC. The first concerns about 85% of all patients and consists of

frequent mutations in *APC*[23], *TP53*[24], *KRAS*[25], *BRAF*, *TTN*, *PIK3CA*[26], *FBXW7*[27] and *SMAD4* genes[28]; the second concerns 15% of CRC-sporadic patients and is characterized by a high level of hypermethylation of the *MLH1* gene, responsible for DNA mismatch repair[29]. Additionally, a different complement of mutations in somatic genes has also been described[30].

Single gene sequencing

Considering their role in resistance to multiple treatment strategies, genotyping of gene mutations currently represents an important diagnostic and therapeutic tool (Figure 2). For instance, a mutation in *APC*, a tumor suppressor gene highly mutated in 57% of CRC cases and involved in DNA replication and repair processes, has been documented to strongly influence the chemotherapy response[31]. Also, *SMAD4* gene mutations were observed in 2%-20% of CRC cases and were usually associated with poor response to cetuximab treatment[32]. In addition, several *RAF* mutations have been implicated in the induction of genomic instability, driving the proliferation of cancer cells[33], while heterogeneous *KRAS* mutations have been identified in almost 40% of CRC patients[34] (with a substitution in the G12C position as the most common detected), having a consequent association with anti-epidermal growth factor receptor treatment resistance[35].

To better represent the cancer heterogeneity using NGS technology, Ye *et al*[36] proposed a protocol for conducting rigorous systematic reviews and meta-analyses on the accuracy of *KRAS* mutation detection in CRC using non-invasive liquid biopsy samples[36]. Generally, liquid biopsies represent the collection of tumor-derived biomarkers in the blood or other body fluids, such as urine, saliva, stool or cerebrospinal fluid. Circulating tumor DNA (ctDNA), circulating tumor cells and exosomes are the most common tumor-related biomarkers assessed on liquid biopsy so far[37]. Moreover, the Food and Drug Administration recently approved a liquid biopsy test to analyze the frequency of *KRAS*, *NRAS* and *BRAF* hotspot mutations in ctDNA that could represent good CRC prognostic factors[38].

Multi-target stool DNA test

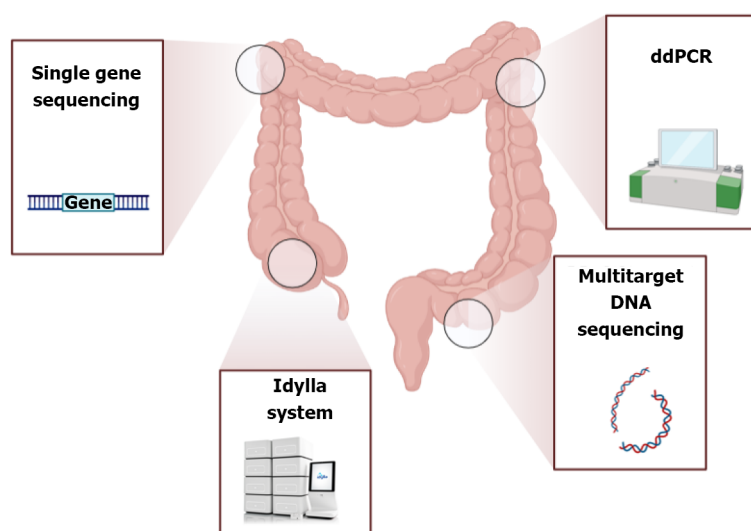
The multi-target stool DNA (MT-sDNA) test allows the identification of specific gene mutations in human tumor DNA cells separately from the more abundant microbial DNA in the stool (Figure 2). During the last few years, several key technological advances have led to increasingly accurate approaches to stool DNA testing including: (1) The use of a DNA preservative swab for stool collection; (2) The improvement of the target capture and amplification methods; and (3) The identification of new informative marker panels[39]. Zou *et al*[40] produced a methyl-binding domain protein bound to a column of nickel-agarose resin to increase the assay sensitivity for detecting methylated DNA markers in stool[40]. Subsequently, multiple prototypes of MT-sDNA test were commercialized, but only two were approved in August 2014 by the Food and Drug Administration for screening people at average risk for CRC aged over 50 years[29]. To date, both the American Cancer Society and the United States Preventive Services Task Force affirmed that the MT-sDNA test can be repeated every 3 years to provide a decrease in CRC incidence and mortality with an acceptable cost and have approved this test for screening people of ages 45 years to 49 years[41,42].

Moreover, Heigh *et al*[43] performed a targeted single assay test with aberrant methylation of *BMP3* alone and detected sessile serrated polyps with a sensitivity of 66% and a specificity of 91%[43]. Although additional biomarkers can be used by including multiple targets that reach the 21-target MT-sDNA test, no increase in the sensitivity or specificity was observed[44]. In general, most studies agree that MT-sDNA is effective to detect CRC with only a few exceptions. In fact, Imperiale *et al*[16] detected 60 out of 65 colon cancers by MT-sDNA test with an estimated sensitivity of 92.3% and a specificity of 90%, confirming that the MT-sDNA test is more sensitive than FIT, especially for the detection of lesions with high-grade dysplasia or sessile serrated polyps (≥ 1 cm). Overall, the method sensitivity varied from 62% to 91% for cancer and from 27% to 82% for advanced adenomas, with a specificity of 93% to 96% in people with normal findings on colonoscopy[45].

The advancement of the genetic knowledge in CRC and their related mutational events would improve the efficiency and the sensitivity of MT-sDNA tests by increasing the target DNA genes. Nowadays, MT-sDNA tests include quantitative molecular assays for *KRAS* mutations, *NDRG4* and *BMP3* methylation and β -actin and include eleven different DNA sequences commonly seen in colon polyps/cancers[46]. Therefore, as confirmed by a retrospective study conducted by Weiser *et al*[47] on 368494 subjects, the MT-sDNA test represents the most recommended CRC screening tool because of its widespread accessibility and higher sensitivity compared with other previously described methods such as FIT and FOBT (Table 1)[47].

Droplet digital polymerase chain reaction

Droplet digital polymerase chain reaction (ddPCR) is recognized as an established and trustworthy approach for clinical cancer research due to its high sensitivity (almost 74% for CRC) in comparison to traditional standard procedures, even in degraded samples[48] (Figure 2). This method consists of an enrichment strategy that allows the detection of low-level mutations by amplification of single DNA molecules without the need for standard reference curves. It is considered much easier, faster and less error-prone than real-time quantitative PCR[49]. Nowadays, ddPCR is commonly used for detecting



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Figure 2 Colorectal cancer screening based on Sanger DNA sequencing. ddPCR: Droplet digital polymerase chain reaction.

rare alleles as molecular markers in plasma samples of pre- and postoperative CRC patients not only because of its high sensitivity for detecting tumor DNA (even with a very small fraction or degraded DNA) but also to monitor disease progression and the emergence of drug resistance[50]. Through this method, Taly *et al*[51] documented seven common mutations in codons 12 and 13 of the *KRAS* oncogene from plasma samples of CRC patients, demonstrating the clinical utility of multiplex ddPCR to screen multiple mutations with a sensitivity sufficient to detect mutations in circulating DNA obtained by non-invasive blood collection[51].

In the same context, ddPCR platforms using OncoBEAM technology demonstrated high sensitivity for plasma detection of *KRAS* mutations[52], and overall ddPCR has been largely applied to the detection and quantification of mutated genes including *KRAS*[53], *BAT26*[54], *ITGA6* and *ITGA6A*[55] and hypermethylated *GRIA4*, *VIPR2*[56] and *VIM*[57] from both ctDNA or fecal DNA of CRC patients. Recently, Garrigou *et al*[58] proposed the screening of modifications in methylated ctDNA as a biomarker to monitor tumor evolution of CRC patients at different stages and concluded that it could be a universal approach to follow tumor burden of CRC patients as compared with mutated ctDNA, which requires previous tumor mutation identification[58]. To summarize, although there are many advantages of ddPCR including the high sensitivity and the large range of target mutations, its major limitation is represented by the lower availability of primer/probe sets (Table 1)[59].

The Idylla approach

The Idylla system (Biocartis, Mechelen, Belgium) consists of a cartridge-based fully automated medical device able to perform an innovative technology that consists of a conventional TaqMan reporter system and novel chemistry known as PlexPCR (amplicons containing a small region with a sequence different from that of target DNA) simultaneously with a PlexZyme (specific amplicon sequence-matched reporter probe) that allows multiplexing of numerous gene targets in one assay[60] (Figure 2). Hence, due to its ability to easily detect a wide range of CRC-related mutations, the Idylla approach can be easily implemented in pathology laboratories to reduce turnaround time[61]. It currently represents a feasible and validated test for *KRAS*, *NRAS* and epidermal growth factor receptor mutations in formalin-fixed paraffin-embedded tissues[62] and for *BRAF* hotspot mutations in plasma samples[63].

In addition, the Idylla system can be used to confirm uncertain outcomes of doubtful NGS results and/or in case of scarce tissue material within a few hours. For instance, Zwaenepoel *et al*[64] evaluated the clinical performance of the Idylla method in 330 CRC samples and demonstrated that this technology was able to give results in less than 2.5 h with only two invalid results. Many authors tested the full panel of CRC gene targets (*BRAF*, *KRAS* and *NRAS*) and found that the concordance between Idylla and NGS was 100% for *BRAF* and *KRAS* mutations and 94% for *NRAS*[65]. Therefore, this methodology is highly accurate for detecting frequent mutations and minimizing the contamination risk, in addition to reducing cost per test when compared with NGS or some conventional PCR assays. However, rare and/or complex genomic variants, which are not included in the reference ranges, cannot be detected by the Idylla system, and continuous improvement of its biomarker panel is necessary to guarantee efficient diagnosis[66].

METHODS BASED ON NGS TECHNOLOGIES

Since the 2000s, and in coincidence with the emergence and development of new high-throughput sequencing technologies, many analyses have been undertaken to examine genetic susceptibility to diseases through genome-wide association studies. Zanke *et al*[67], using a multistage genetic association approach comprising 7480 CRC patients and 7779 controls, recognized a wide association of markers in chromosomal region 8q24, the same site where the *SMAD7* gene is located[67]. In addition, a genome-wide association study performed by Broderick *et al*[68], consisting of the genotyping of 550163 tag single nucleotide polymorphisms in 940 individuals with familial CRC and 965 controls, identified three single nucleotide polymorphisms in the *SMAD7* gene[68]. Subsequently, Tomlinson *et al*[69] confirmed these results and elucidated other markers in chromosomal regions of 8q23.3 and 10p14 at which common variants can influence the risk of CRC development[69].

NGS-based diagnostic assays are increasingly adopted especially with decreasing sequencing costs. In the early stage, sequencing technologies were used to target driver genes known to contribute to CRC, but recently larger chromosomal regions have been targeted exploiting the potential of these technologies in multigene sequencing by using a very low amount of biological material from liquid or tissue biopsy samples. In this step, many efforts have been made to standardize sequencing procedures and data analyses and to generate databases that store the sequencing information. Clinicians and research communities can use this information to provide better quality care[70].

Early in 2010, The Cancer Genome Atlas project conducted a genome-scale analysis of samples obtained from 276 CRC patients, analyzed exome sequences, DNA copy number, promoter methylation and messenger RNA and microRNA expression and concluded that 16% of CRC samples were found to be hypermutated, 77% of patients displayed one or both breakpoints leading to translocation in an intergenic region and 7% of patients reported a translocation involving the *TTC28* gene (an inhibitor of tumor cell growth) located on chromosome 22[71]. Furthermore, the Pan-Cancer Analysis of Whole Genomes, the International Cancer Genome Consortium and The Cancer Genome Atlas projects recently described 2658 whole genomes of tumor samples and their matching normal tissues, not only of CRC but of 38 different cancer types, providing insights into the nature and timing of the many mutational processes that shape large and small-scale somatic variation in the cancer genome[65].

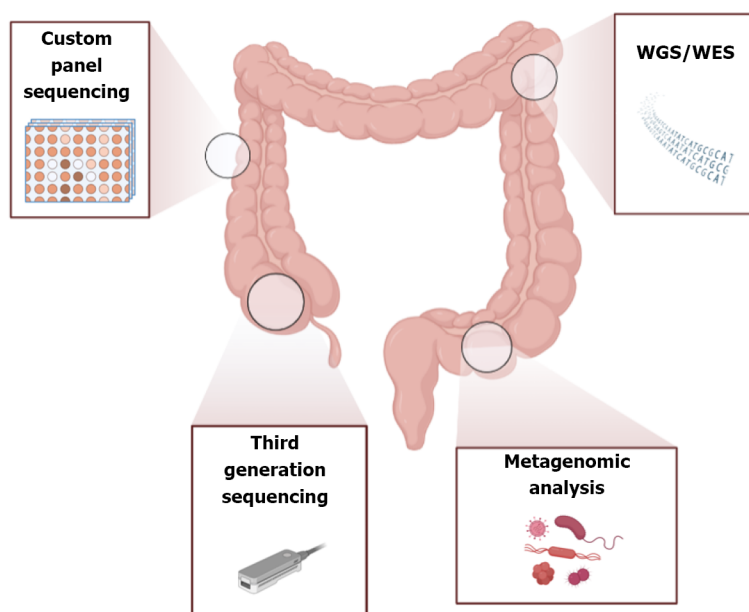
According to the improvement of NGS approaches, different sequencing platforms have been developed (Illumina, Ion Torrent, SOLiD, PacBio and Nanopore) that are classified in terms of maximum output, reads per run, accuracy, run time, amount of nucleic acids necessary for analysis and reads length. In particular, they can generate short (*e.g.*, SOLiD, Ion Torrent, Illumina) or long reads (*e.g.*, PacBio, Nanopore). While short reads sequencing does not exceed 300 base pairs and is more suitable for CRC diagnosis, long reads sequencing determines a better coverage of the genome and is more adaptable for large deletion/insertion determination or chromosomal rearrangement[72]. Considering that both short-read and long-read sequencing have their benefits and flaws depending on the experimental aim, it is important to remark that when somatic alterations in oncogenes and tumor suppressor genes are stable throughout the tumor clonal evolution, chromosomal alterations and copy number variation (CNV) could be lost during cancer progression[73].

In addition, CRC represents one of the most interesting fields of NGS application because of its great quantity of activating mutations; in fact, next-gen techniques enable the identification of novel mutations/ altered genes or genomic rearrangements allowing the discovery of new possible treatments [74]. In general, there are three more common NGS-based methods used for CRC studies: Custom panel; whole genome sequencing (WGS); or whole exome (WES) sequencing and third-generation sequencing approaches. In general, large-scale mutations were identified by WGS of tumor DNA, while point mutations were identified by targeted sequencing (Table 1).

Custom panel sequencing

During the last decade, several pipelines based on NGS approaches have been developed, and additional somatic mutations and chromosomal aberrations were detected in CRC samples (Figure 3). To simplify routine adoption of NGS tools, Zheng *et al*[75] considered a custom-designed panel of genes of only 2.2 Mb (exons and partial introns of cancer driver of more than 600 genes) and deduced a 9-loci model for detecting microsatellite instability (MSI) with 100% sensitivity and specificity compared with MSI and 84.3% overall concordance with immunohistochemistry staining[75]. Many authors have undertaken the simultaneous sequencing of many driver genes including low allele frequencies using NGS technologies and have emphasized the importance of the fine classification of mutational status as some cancers were associated with poor prognosis treatment[76]. In this regard, the comprehension of the wide heterogeneity of CRC lesions seems to be an extremely important point for tracing the therapeutic approach of the patient and developing effective strategies for early CRC detection and prevention.

Liquid biopsy samples have been investigated more than tumor tissue samples because of their non-invasiveness and their better representation of cancer heterogeneity[77]. In this context, Myint *et al*[78] developed a multiregional NGS approach from circulating cell-free DNA using a customized targeted CRC panel consisting of all coding exons of 116 genes, 22 genes recurrently amplified/deleted, 51 copy number regions, 121 MSI regions and 2 gene fusions (*RSPO2* and *RSPO3*) and confirmed the



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Figure 3 Next-generation sequencing-based colorectal cancer screening. WES: Whole exome sequencing; WGS: Whole genome sequencing.

widespread genetic heterogeneity in six adenoma samples, which affected the driver genes *MMR*, *APC*, *PIK3CA*, *TP53* and *SMAD4*[78]. Additionally, based on an NGS analysis of a panel of 324 CRC-associated genes, Stahler *et al*[79] documented frequent single nucleotide variations in the *TP53*, *APC*, *KRAS*, *PIK3CA*, *BRAF*, *SMAD4* and *FBXW7* genes, and copy number alterations in the *MYC* and *FLT3* genes[79].

Furthermore, Leary *et al*[80] developed a “personalized analysis of rearranged ends” approach, which can identify translocations and copy number alterations in CRC and other solid tumors. In addition, personalized analysis of rearranged ends can detect 57 regions containing putative somatic rearrangements, with an average of 14 rearrangements per sample[80]. Moreover, targeted sequencing strategies based on short reads and CNV determination could represent a good strategy for CRC studies. In fact, Gould *et al*[81] confirmed that an NGS approach using short fragments presented a sensitivity > 96% and a specificity > 99% for detecting samples with CNVs in the terminal five exons of *PMS2*[81].

Additionally, Corti *et al*[82] developed multiple DNA NGS approaches coupled with the computational and bioinformatics algorithm “IDEA” to target a WES of about 30 Mb, a custom panel of genes of about 603 Kb (frequently mutated genes) and another of 918 Kb (intron-exon junction to precisely identify the genomic breakpoint)[82]. Currently, IDEA represents a flexible and comprehensive pipeline for the management of CRC patients and is suitable for identifying several genetic alterations from a non-invasive sample (ctDNA) such as single nucleotide variants, insertions and deletions, gene copy-number alterations and chromosomal rearrangements in the *KRAS*, *BRAF*, *PIK3CA* and *ERBB2* genes (usually involved in drug resistance). In general, sequencing of smaller target regions provides greater sequencing depth which allows for better recognition of low gene frequency variation. Hence a customized gene approach is more suitable for clinical oncology laboratories for many advantages such as the simplicity, low cost and fast of the method and the non-need for bioinformatics specialists in the laboratories (Table 1).

WES and WGS

The contribution of MSI to the tumor mutational burden (TMB) due to a defective mismatch repair system is considered important in about 15% of CRC patients. According to the phenotype, MSI tumors can be divided into two distinct MSI phenotypes: MSI-high and MSI-low[83]. Recently, considering that the defective mismatch repair phenotype is crucial to define the efficacy of immune checkpoint inhibitor treatment, Xiao *et al*[84] used WES to evaluate the immune microenvironment and 2539 microsatellite loci in a group of 98 CRC patients. They concluded that the microenvironment of TMB-high was significantly more immune-responsive than TMB-low[84]. On the other hand, Gurjao *et al*[85] demonstrated the presence of a novel alkylating mutational signature, identified through the WES of 900 CRC patients and predicted that *KRAS* p.G12D, *KRAS* p.G13D and *PIK3CA* p.E545K driver mutations were mainly targeted by the alkylating signature in non-hypermutated patients[85].

Moreover, Chang *et al*[86] performed the WES of DNA obtained from tumor tissues of 32 surgical CRC patients and identified the well-known recurrent mutations in the *APC*, *TP53*, *KRAS* and *FBXW7*

genes and unreported mutations in additional 14 genes[86]. Furthermore, many authors confirmed that WGS largely contributed to determining the significant role of non-coding regions such as enhancers, transcription factor binding sites, promoters and 3'untranslated regions in CRC carcinogenesis[87]. In addition, WGS was used to demonstrate that metastatic lesions were enriched in gene mutations affecting PI3K-Akt signaling, cell adhesion and extracellular matrix processes[88]. Finally, Dashti *et al* [89] conceived a new technique based on a novel concept called 'gene-motif,' which identified seven CRC subtypes that can be effectively used to develop a personalized treatment[89].

In comparison to WES, the WGS approach has the advantage of increasing the overall variant accuracy and poor coverage but is more expensive and requires fresh-frozen tumor material to perform analysis of the highest quality (Figure 3 and Table 1).

Third-generation sequencing

Third-generation sequencing of long reads has been developed and represents the most suitable approach for the identification of deletion/duplication breakpoints and complex structural variants and CNV-neutral rearrangements such as inversions and large intronic insertions[90] (Figure 3). Indeed, many studies affirmed that long-read sequencing technologies have potential advantages over existing alternatives especially when pathogenic variants are in complex genomic regions, such as the recurrent *PMS2* insertion-deletion variant. Using a locus-specific amplicon template, Watson *et al*[91] undertook Nanopore long-read sequencing to assess the CRC diagnostic accuracy of this platform. Pairwise comparison between sequencing results derived from short-read NGS and unidirectional Sanger sequencing and the consensus Nanopore dataset revealed 100% sequence identity[91]. Furthermore, reads produced by Nanopore oxford technology were able to identify both the 5' and 3' junctions and revealed detailed insertion sequence information[92].

METAGENOMICS ANALYSIS OF GUT DYSBIOSIS IN CRC PATIENTS

Genetic factors that concern somatic mutations in *KRAS*, *APC*, *p53*, mismatch repair genes and other chromosomal aberrations explain less than 35% of all diagnosed CRCs, and many environmental exposures seem to modulate the cancer risk[93]. For instance, metagenomics studies based on 16S rRNA sequencing that has been recently conducted have documented the presence of more than a thousand microbial species in the human gastrointestinal tract carrying more than 100 times as many genes as the human genome[94] (Figure 3).

Therefore, considering the high microbial diversity in humans and their contribution to host health and pathological or malignant conditions, it was suggested that about 20% of the global cancer burden can be linked to microbial agents[95]. However, in addition to the several factors that can considerably modify the gut microbiota (GM) composition (*e.g.*, age, sex, nationality, dietary and lifestyle habits, drugs or alcohol abuse)[96], multiple experimental challenges can influence the results of GM studies such as sampling methods and consistency[97], storage sample conditions[98], DNA extraction methods [99], type of primers used and pipelines adopted for data analyses[100]. For all these reasons, it is very hard to define a baseline microbial community for healthy people, especially due to the impossibility of obtaining biopsy samples from healthy controls. Therefore, tumor-adjacent tissue has been regarded as the healthy control, but many efforts have recently been expended to standardize the experimental and analytical methods[101]. The two most common metagenomics approaches for GM characterization are shotgun sequencing and metabarcoding.

These NGS-based approaches both contain three basic steps: Library preparation; sequencing; and data analysis. Sequencing libraries are typically created by fragmenting DNA and adding specialized adapters to both ends to allow the DNA fragments to bind to the sequencer flow cell. Due to unique barcodes added to each library that are used to distinguish between the libraries during data analysis, multiple libraries can be pooled together and sequenced in the same run (a process known as multiplexing). During the next sequencing step of the NGS workflow, the sequencer amplifies the DNA fragments, resulting in millions of copies of single-stranded DNA. In detail, chemically modified nucleotides bind to the DNA template strand through natural complementarity, and each nucleotide contains a fluorescent tag and a reversible terminator that blocks the incorporation of the next base. The fluorescent signal indicates which nucleotide has been added, and the terminator is cleaved so the next base can bind. After reading the forward DNA strand, the reads are washed away, and the process repeats for the reverse strand. After sequencing, the instrument software identifies nucleotides (a process called base calling) and the predicted accuracy of those base calls. Finally, data analysis can be performed with standard tools or with customized techniques[102].

While shotgun sequencing results in a very complicated data output (*i.e.* a huge amount of information that can be up to 1.5 terabases per run) because it simultaneously provides functional and taxonomic information about bacteria, fungi, viruses and a variety of other microorganisms, metabarcoding has a less complex data output and provides only taxonomic information about the bacterial (16S region sequencing) or fungal (ITS sequencing) composition of the sample (Table 1)[103]. Thus, metagenome-wide association studies have identified a correlation between many microbial species/

gene markers and CRC, promoting the development of an affordable diagnostic test using both stool and tissue samples[104,105].

CRC-associated bacteria

Much evidence has documented GM involvement in different diseases, including CRC. In particular, recent reports have demonstrated a bacterial driver-passenger model for CRC initiation and progression and showed that the first epithelial transformations can be supported by certain intestinal bacteria[106]. In 2012, Tjalsma *et al*[107] proposed a bacterial driver-passenger model for CRC in which pathogenic driver bacteria interact transiently with host cells to initiate CRC development and are then replaced by other passenger bacteria species that were unable to colonize healthy colon tissue but benefitted from altered metabolism of tumors cells[107]. To date, Wang *et al*[108] have identified *Bacillus spp.*, *Bradyrhizobium spp.*, *Methylobacterium spp.* and *Streptomyces spp.* as potential driver bacteria and *Fusobacterium spp.* and *Campylobacter spp.* as certain and abundant passenger bacteria[108].

Moreover, Luan *et al*[109] characterized the mucosa-adherent fungal microbiota of paired biopsy samples of adenomas and adjacent healthy tissue from 27 subjects using barcoded high-throughput sequencing that targeted the ITS region and reported a different fungal composition in patients with different adenoma stages and identified the phylum Glomeromycota as a possible powerful CRC marker[109]. Consistently, recent findings obtained through the WGS approach demonstrated that the Ascomycota/Basidiomycota ratio could represent a potential novel marker for early CRC detection [110]. Furthermore, Coker *et al*[111] used a shotgun metagenomics approach to evaluate the role of the archeome in colorectal carcinogenesis and found distinct archaea clusters in fecal samples from CRC patients, patients with adenomas and healthy subjects, with the CRC patients showing significant enrichment of halophilic archaea and depletion of methanogenic archaea[111].

Several metagenomic analyses of CRC patients have documented an over-representation of *Fusobacterium nucleatum* (*F. nucleatum*) in both tissue or stool samples in comparison to healthy controls[112]. Interestingly, in a large cohort of 616 participants, Yachida *et al*[113] demonstrated that the shift in the GM composition between CRC patients and healthy controls occurred in the very early stages of CRC development. In particular, the relative abundance of *F. nucleatum* was significantly elevated continuously from intramucosal carcinoma to more advanced stages, while *Atopobium parvulum* and *Actinomyces odontolyticus* were significantly increased only in multiple polypoid adenomas and/or intramucosal carcinomas[113]. Recently, in addition to *F. nucleatum*, several bacteria, such as *Bacteroides fragilis*, *Escherichia coli*, *Streptococcus bovis*, *Enterococcus faecalis*, *Peptostreptococcus anaerobius* and *Lachnospirillum spp.*, have been reported to be enriched in stool or tissue samples of CRC patients compared to healthy ones[114-118].

Moreover, an association between specific bacterial species and antitumor responses have been reported; for instance, a positive correlation between the abundance of *Bifidobacterium longum* or Ruminococcaceae members and the efficiency of CRC immunotherapy has been documented[119]. *Eubacterium limosum*, *Ruthenibacterium lactatiformans*, *Fusobacterium ulcerans*, *Bacteroides uniformis*, *Paraprevotella xylaniphila* and *Alistipes senegalensis* improved the effectiveness of immune checkpoint inhibitors[120].

Because the GM composition can be modified by probiotic and prebiotic supplementation, which can help maintain intestinal microbial homeostasis and mitigate dysbiosis, many reports have evaluated their effect on colorectal carcinogenesis. Overall, recent systematic reviews suggested that prebiotics may have a protective effect on the progress of CRC, while the administration of certain probiotics in patients with CRC reduced the side effects of chemotherapy, improved the outcomes of surgery, shortened hospital stays and decreased the risk of death[121,122]. However, the findings are still conflicting, and none determined changes in bacterial richness and diversity that are usually reduced in CRC patients. Thus, further studies are needed to better understand the prebiotic and probiotic effects in CRC patients.

CRC-associated bacterial metabolites

Accumulating evidence has suggested that GM modulates the CRC progression, and its metabolites can play a crucial role in this scenario. The rapid development of technologies such as mass spectrometry and nuclear magnetic resonance have documented different profiles of microbial metabolites between CRC patients and healthy subjects. For instance, lower bile acid hydrolase and β -galactosidase abundances and higher levels of leucine, tyrosine, valine, choline, colibactin, gallocin, formyl methionyl leucyl phenylalanine, *Bacteroides fragilis* toxin and trimethylamine-N-oxide have been associated with CRC development[123-125]. Furthermore, the total amount of short chain fatty acids, the main metabolites produced by the bacterial anaerobic fermentation of indigestible polysaccharides that exert various and fundamental functions for the host, was significantly lower in fecal and plasma samples of CRC patients compared to both patients with adenomatous polyps and healthy controls. Therefore, these metabolites could represent novel potential non-invasive diagnostic biomarkers for CRC[126,127]. In addition, recent investigations have reported that the GM plays a critical role in the effectiveness of anti-CRC treatments, including chemotherapy as well as immunosuppressive agents. For instance, it has been reported that the effectiveness of CRC treatment with 5-fluorouracil is enhanced by certain microbial metabolites[128]. The supplementation with probiotics or prebiotics could increase chances of

therapeutic success^[129].

CONCLUSION

Since the advent of NGS approaches, many molecular techniques for the diagnosis of CRC from invasive or non-invasive sampling have emerged and have significantly increased the number of known genes and mutations linked to CRC. However, due to the multitude of host and microbial genetic factors and the complexity of the tumor environment, the optimization of a CRC biomarker remains difficult, especially in stool samples, in which the complexity of the lesion environment seems to play a key role^[50]. Thus, the development of a biological method to find stable, sensitive and specific markers in non-invasive samples such as feces or plasma remains an arduous challenge to be carried out. Furthermore, despite the great progress in metagenomics methods and bioinformatics tools, WES and WGS are still feasible only in expert centers. Only limited pieces of genomic information are currently clinically relevant for the care of CRC patients, and the list of predictive actionable genomic biomarkers is quite short^[86]. Apart from the identification of novel microbial biomarkers, new CRC-associated molecules are under evaluation for CRC screening, such as circular RNA and Piwi-interacting RNA. These advances in the identification of microbial markers and the improvement of non-invasive diagnostic capabilities and their applications in guiding precision cancer therapies are poised to change the diagnosis of CRC and select and monitor the treatments in the near future due to the increasingly adopted precision medicine for the care of CRC patients.

FOOTNOTES

Author contributions: Abbes S and Baldi B contributed to the conceptualization and revision of the literature; Abbes S contributed to the original draft preparation; Sellami H, Amedei A and Keskes L contributed to critical revision and supervised the manuscript; Abbes S and Baldi S wrote the review and edited; Amedei A and Keskes L contributed to the funding acquisition; All authors have read and agreed to the published version of the manuscript.

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