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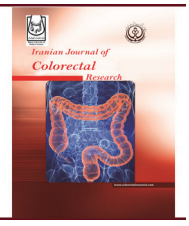

Novel Molecular Prognostic and Diagnostic Techniques in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is a prominent cause of malignancy-associated death worldwide. This disease is predominantly symptomless as it advances to the highest stages, meaning that screening schemes aimed at early diagnosis are required to lower the prevalence and fatality rate. We aimed to review the literature on different molecular procedures for detecting stool-based biomarkers of CRC.

We reviewed papers from Google Scholar and PubMed on different molecular procedures for detecting stool-based biomarkers of CRC with no time limitation.

An integrative framework of all epigenetic and genetic modifications was studied, representing more acceptable specificity and sensitivity for the diagnosis, treatment, and drug response/outcome evaluation of CRC compared to other traditional procedures. Differential expression analysis of stool-derived RNA (sRNA) and stool DNA (sDNA) testing for ultrasensitive mutations, methylation, and fragmentation patterns can lead to an accurate, early-stage diagnosis and a better prognosis for CRC patients.

Keywords: Colorectal cancer, DNA, Feces, Screening, Diagnosis, Prognosis

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Introduction

Colorectal cancer (CRC) is relatively common and on the rise worldwide in both sexes, especially in countries with high rates of saturated fat intake and cigarette smoking (1). To minimize the mortality caused by CRC, the fecal immunochemical test (FIT) is recommended for screening in many countries, though colonoscopy remains the gold standard for screening in the USA and Germany (2-4). Contrary

to non-invasive testing, invasive procedures like flexible sigmoidoscopy and colonoscopy facilitate the conduction of polypectomy to remove premalignant injuries, reducing the prevalence and fatality of CRC (5). Regardless of the decrease in CRC prevalence and fatality with screening, screening measures still need improvement as some cases are missed (6, 7). As an invasive screening measure, colonoscopy is underutilized due to the costs involved, procedural pain, limited service providers, and the need to schedule the procedure, prepare the bowel, and cease working (8, 9).

When examining the advantages of invasive vs. non-invasive screening tools for colorectal tumors, it is essential to remember that discovering remediable-stage tumors will feasibly diminish CRC mortality; precancerous lesions can be removed, leading to a lower CRC frequency (10). For example, the yearly guaiac fecal occult blood testing (gFOBT) reduces CRC fatality by nearly 14% (compared with no screening), but it does not lessen CRC occurrence (11, 12). A recent study surveyed the findings of CRC screening schemes in Europe; for an affirmative FIT, the prognostic value for progressed polyps ranged from 5–30% (13). However, serrated adenomas larger than or equal to 1 cm are rarely diagnosed by FIT screening.

Novel non-invasive screeening tests include multi-target stool DNA (MT-sDNA) (Cologuard®, Exact Sciences Corporation, Madison, WI), methylated NDRG4, SEPT9, and SDC2 assays, as well as the DNA mutation panel (point mutations in K-Ras, APC and p53 genes; microsatellite instability marker BAT-26 deletions; and long DNA assay). MT-sDNA uncovers remediable-stage colorectal malignancy with 93–100% sensitivity and outperforms FIT in the diagnosis of progressed polyps and serrated precursor lesions, sharing an association with the risk of development to tumor (14-17). MiRNAs are strongly linked with infectious bowel diseases; a new survey has exhibited their function in controlling inflammatory reactions and gastrointestinal disorders. Stool miRNA is linked with intestinal barrier malfunction, which can induce inflammatory bowel disease (IBD)—a condition associated with CRC. Hence, this biomarker is a strong biosignature for checking and verifying CRC with high specificity and sensitivity (18, 19) (Table 1). We aimed to review the literature on different molecular procedures for detecting stool-based biomarkers of CRC. Advances in molecular biomarker research and the innovation of accurate non-invasive assays facilitate improved CRC screening, diagnosis, and prognosis prediction, resulting in better patient outcomes.

Experimental Approaches for Extraction, Detection, and Assaying of Stool DNA and RNA

Although basic experiments and clinical investigations have shown the importance of DNA and RNA biomarkers in CRC diagnosis or prognosis, but their practicability need to be evaluated. In addition, stability and persistence are crucial features of tumor biosignatures, as the specimen must remain viable during transfer and storage ahead of testing (23). Fecal specimens provide a noninvasive solution to exfoliate colonic epithelial cell indicators of CRC incidence. Nevertheless, one of the most serious difficulties in population-based research on stool indices is collecting sufficient tests from those who suffer from CRC (24, 25). This problem is worsened because usual stool-gathering systems deal with fresh or frozen specimens that restrict their use in communal processing. Nucleases in feces can quickly break down, so estimates must be made to counter their effects. As this issue can affect human genomic DNA and block PCR, diagnostic assays were planned to identify, compute, and determine the background over which this bias is the lowest (26-28). Surveys expressed that specimens gathered in a fixation dilution (Buffer EDTA 20 mM, Buffer EDTA 100 mM, RNAlater RNA Stabilization Reagent (Qiagen); Cytolyte (ThinPrep, Cytyc Corporation); Buffer Genefec (NORDIAG); Buffer α-Wasserman and refrigerator storage) increase DNA durability and entirety in a way that undamaged nucleic acids symbolic of intact cells remain identifiable, with the best results achieved with the 100 mM EDTA buffer frozen after 24 hours (29). The PCR multiplications' findings showed that DNA multiplication was

Table 1: Currently recommended non-invasive approaches for CRC screening

Fecal-based test	Repetition	Indication of validity	Sensitivity	Specificity
FIT	Yearly	Systematic review and meta-analysis studies exhibit superior sensitivity vs. gFOBT	79%	94%
gFOBT	Every 1–2 years	Valuable non-invasive advantages compared to10-yearly colonoscopy along with low sensitivity for proximal colonic injuries detection. Meta-analysis indicate a low mortality rate of up to 14% in average-risk populations (20)	72.2%	90%
MT-cDNA	Every 3 years	Valuable non-invasive screening assay compared to FIT for primary-stage CRC and advanced polyps originated from right or colonic injuries (21).	85%	90%
MT-sRNA	Not Established	Multimarket fecal RNA preferable to FIT or FOBT for evaluating proximal CRC by low quantity of required sample (22)	29–90%	75–95%

FIT: Fecal immunochemical test, gFOBT: Guaiac fecal occult blood testing, MT-sDNA: Multi-target stool DNA, CRC: Colorectal cancer, MT-sRNA: Multi-target stool RNA

considerably lowered in specimens, although specimens kept in fixation buffer provided increased band intensity.

Similarly, the addition of buffer to specimens before environmental temperature incubation resulted in an important rise in DNA constancy and rigidity with no PCR blockage (because of the presence of various factors such as hemoglobin breakdown output, bile salts, and complicated polysaccharides), compared to the corresponding specimens without buffer (30-33). The quantitative evaluation of stool DNA emphasized multiple concerns crucial for additional studies in this scope. Initially, the maximum sensitivities were achieved when the duplicates were small, ideally lower than 100 bp. This is due to DNA degradation in tumor cells that experience necrosis or apoptosis before or after secretion into the stool because proximal section of colon with a hydrated milieu induce DNA destruction more compared to nucleases and enzymatic hydrolysis (34-36) while colonic distal sections with more hydrated enviroment will make a very good milieu to conserve the DNA (37). To offset for dissimilatory in hydration conditions, the waterless mass should be specified, for instance, by freeze-drying. While optimal for the conservation of the DNA, freeze-drying is time-taking, effortful, and not achievable in all laboratories. An additional confusing factor has been the necessity for quantitative isolation of human DNA from every portion of fecal samples. Consequently, to overcome this obstacle, we can measure the quantity of human DNA in relation to the quantity of whole DNA. As fecal specimens are multiplex in constitution, sampling errors caused by testing small fractions can be avoided by testing different fractions from every fecal specimen. The pre-handling characteristics of fecal container specimens control DNA efficacy, and an appropriate DNA extraction kit (e.g., the QIAamp DNA Stool Mini Kit) must be used (37).

Difficulties in eradicating PCR blockers from fecal samples have been thoughtfully announced, and, for various cases, the dilution of specimens affirms the necessity to supply a fast and direct procedure for multiplication. Although, dilution is just workable if the extent of DNA is adequately great. Thus, for utilizations containing low-copy objectives and contaminating bacterial DNA, the dilution solution is usually unfavorable and occasionally inconceivable. Other techniques have been applied to lighten the impacts of restrictive materials of PCR, like the utilization of segregation columns (38, 39). They introduce several difficulties, such as lower DNA outputs, fewer DNA targets, and reduced amplification potential.

Spermidine, a polyamine synthesized, is reported to possess a high affinity for plant and fecal DNA, making it useful as a PCR promoter. It is assumed that spermidine may obstruct the activity of PCR blockers (probably through attaching them and or producing them more thermolabile, or, preferably, attaching to DNA at small concentrations may significantly reduce the activity of the PCR blockers). Beyond a specific concentration, spermidine no longer restricts PCR blockers, probably because of steric influences or saturation, and begins extensively attaching to DNA, thereby suppressing PCR. Noteworthy, the addition of spermidine drives a favorable trend of the melting temperature of the fecal and normal DNA and aids the multiplication productivity of methylation indexes by means of the Quantitative Multiplex Methylation-Specific PCR (QM-MSP). Spermidine adding is simpler and more applicable than dilution or filtration techniques and may remarkably ameliorate the assessment of methylation rates (in the proximity of 1 mM spermidine and 100 ng of fecal DNA).

Mutant DNA constitutes a small proportion (median, 0.32%; mean, 1.89%) of the whole DNA from stool specimens of tumor patients, limiting its effect on quantifying the integrity of the normal and mutant DNA. The discovered enhancement of DNA stability in tumor cases arises from the secretion of bigger DNA pieces from control cells across the cancer milieu into the stool flow (40). These tumors are commonly penetrated by specific inflammatory cells, which might donate large DNA fractions of the control sequence. Hence, minimal DNA template molecules are needed to understand the sensitivity of BEAMing (41). The sensitivity of BEAMing for each tested mutation is so that a minimum single mutant pattern be sensed within 10,000 control patterns (0.01%). For many mutations, the sensitivity is as great as a single mutant pattern enclosed by 800,000 healthy patterns (0.0013%) (42). The sensitivity is confined exclusively via the miss rate of the polymerase employed in the first amplification. The application of this more-tech sensitivity in the procedure calls for a sufficient amount of DNA templates. For instance, if just 2000 scheme molecules are applied to each test, the highest sensitivity is 0.05% instead of 0.01%. Getting this amount of patterns is not difficult for fecal specimens but is commonly difficult for plasma (43). The upper sensitivity of BEAMing measuring mutations in feces versus plasma is caused by the proportional parts of mutations detected in the stool of cases with various-stage cancers. However, feces supply an almost unlimited stock of DNA, and additional methodical problems influence the method outputs. For instance, feces include a range of PCR blockers and bacterial DNA, demanding sequence-specific traping of human genomic DNA (43). Sequence-specific DNA fractions were filtrated from the whole nucleic acid provided by performance oligonucleotide-installed hybrid traps (magnetic bead-based sequence-specific). Every traping step was performed by adding guanidine isothiocyanate mixture (GITC), (GIBCO, Invitrogen, Carlsbad, CA), including biotinylated sequence-

specific oligonucleotides and Streptavidin-tagged magnetic beads to the specimen and subsequently the washing of the bead/hybrid trap systems including the sequence-specific traping of DNA with TE over thermal denaturation. Objective human DNA fractions were also detached from absolute nucleic acid sequences using electrophoretical force through a capture sheet which comprised from human sequence- specific trap located inside an acrylamide matrix (44, 45). Trap excavators were polymerized in the form of 37-mer oligonucleotides under 5′-Acrydite alterations (Integrated DNA Technologies, Coralville, IA) (43). However, these assays have not yet been developed to purify small DNA fractions that embrace the target's mutations. The conclusion is that mutant parts as a subordinate of size presumably differ under the specific mutation in a case-particular pattern, which relies both on the healthy DNA's origin and the amount of breakdown of the cancer DNA pieces. This matter may influence the outputs via two paths. First, it likely accounts for the vast discrepancies between the conclusions of mutant parts detected over two genes in two cases. Second, it would describe the reason they were incapable of measuring mutations in many cases.

Enhancement of the trap materials might later raise sensitivity over the 92% rate achieved through methylation-based BEAMing in the Diehl et al. study (46). BEAMing was named after its components (beads, emulsions, amplification, and magnetics); it transforms unique DNA template molecules to individual beads involving tens of thousands of precise transcripts of the template. The higher sensitivity of measuring mutations in feces versus plasma is because of the comparative deductions of mutations found in the stools of subjects with various grade cancers (42).

Quantitative real-time PCR (qPCR), microarray, and next-generation sequencing (NGS) are the more frequently performed assays for estimating fecal-established miRNA. MiRNA varies from mRNA because sequences are short (almost 22 nucleotide) and are usually less frequent. Therefore, qPCR assays should prevent primer dimers and guarantee a small measuring threshold. Recently, two techniques have sought to overpower these problems: stem-loop RT and locked nucleic acid (LNA) primers (47, 48). These primers feasibly ameliorate miRNA identification sensitivity and specificity across linear primers through spatial limitations, base-stacking, and raised thermal constancy. Although the LNA-based assays have performances analogous to those of stem-loop primers, further assessments are required, and specificity could be an issue (49).

To determine new biosignatures for CRC screening, microarray has been broadly accustomed to calculating total human miRNA in stool and blood specimens. Numerous microarray platforms have been prepared for miRNA estimation, involving SurePrint (Agilent), GeneChip (Affymetrix), and miRCURY LNA (Exiqon) (50). Every platform has schemed probes particular for mature miRNA sequences, while chief dissimilarities include attaching and filtration processes besides fluorescent dyeing. Microarray techniques may be utilized to detect various miRNAs concurrently; however, different shortcomings restrict their practicability in medicine (51). Moreover, result standardization is complicated and time-taking, and no individual procedure has been commonly affirmed to calculate microarray results, notably miRNA, because of the minute quantity of miRNA and poor expression status. The normal roadmap includes RNA extraction, library construction, sequencing, and input evaluation. Library construction includes 5′ and 3′ adapter binding and enlargement (52). Adapters are program-accurate and produce a bar code that is admitted throughput multiplication via either bridge PCR (bPCR) or emulsion PCR (emPCR). Compared with the Sanger sequencing platform, NGS is not restricted by the practice of gel or polymer segregation media and hence permits various specimens to be driven simultaneously (53, 54). NGS is perfect for new indicators because it can cross-examine the whole genome. Nonetheless, challenges and limitations also exist. Similar to microarray, NGS result calculating is sophisticated and not normalized. Multiple surveys have cited NGS in blood specimens as beneficial to spot desired miRNA for CRC screening; however, these surveys are mostly certified by another better-focused platform such as qPCR (55, 56).

Fecal-founded approaches are reviewed as the greatest profitable type for plenty of motive. Based on straightforward histological inspections, CRC and polyps shed several humoral cells and their residues within the mucocellular layer covering the colonic lumen. The noticeable molecular alterations induced by CRC cells are reportedly introduced in the feces sooner than in the blood (57). The real superficial zone of the epithelial single-coat of tumors and neoplasia is likely 200 times bigger than expected, along with macroscopic results. Nevertheless, against ample cellular shedding from gross surface zones and neoplastic cells in the mucocellular regions, shed colonocytes scarcely stay alive if they are exfoliated in the right colon owing to intra-luminal lysis. Accordingly, following cell lysis, the measurement of components of the shed cells, like DNA, miRNA, and proteins, is imaginably valuable (58, 59).

Stool DNA/RNA-based Investigations

The recognition of CRC-specific DNA indicators in feces has been discussed comprehensively. The recognition of fecal RNA indicators has not been as widely discussed as DNA indicators because fecal RNA is more unstable than fecal DNA. DNA indicators are distinctive as long as they immediately originate from cancerous cells (49, 60). Methodological approaches in RNA conservation

buffers have progressed to this step to survey CRC cancer-particular RNA transcripts in the form of fecal biosignatures. The exclusively fecal DNA test marketed in the US is ColoSure (Laboratory Corporation of America, https://www.labcorp.com), which offers a 53–83% specificity of CRC detection based on measuring vimentin methylation (61). The implementation of such biomarkers in measuring progressed polyps has not still been outlined, although the sensitivity and specificity for CRC are 72.5–83% and 53–86.9%, respectively (61). Importantly, upregulated copies of mRNA transcripts including MMP7 and PTGS2 are considerably specific to colorectal tumors. CRC gene expression patterns (transcriptomics) and untranslated RNA expression patterns like miRNAs have recently been assessed to distinguish applicant transcripts and study their viable uses in the form of CRC exposure tools (62). Recently, Link and coworkers indicated elevated preciseness of miRNA isolation and expression tests in fecal specimens where miR-106a and miR-21 were constituted to have a great expression in CRC or adenoma cases compared with controls (63).

Genetic Indicators of CRC in Stool

Detection of Genetic Variations

According to the tumorigenesis model of CRC development, primary tumors most commonly constitute massive quantities of intestinal cells, which abundantly stay inside the mucocellular layer of the colorectal mucosa. In individuals with CRC, tumor cell-derived factions of the mucocellular layer are excreted with waste material; these exfoliated cells are non-apoptotic neoplastic colonocytes, contrary to healthy mucosal epithelium exfoliated cells that stem from apoptotic processes (64). The colorectal carcinogenetic pathway can be triggered by repeated genetic mutations and/or epigenetic alterations that lead to continuous evasion of physiological apoptosis (65). Subsequently, such non-apoptotic dysplastic colonocytes following exfoliation from lesioned mucosa and preservation in the stool can secrete undamaged long-DNA (L-DNA) fragments as long as 200 bp or more for testing cancer-corresponding DNA alterations (66, 67). Isolation of cell-arising DNA, RNA, and protein for fecal biomarkers detection and analysis is another feasible and appealing method for early detection and screening of colorectal cancer (25, 68). Various methodological achivements lead to precise fecal DNA measurment assays by employing DNA supportive buffer during feces gathering, capable polymerization techniques, and specific multi-biomarker platforms. A pilot study investigated mutations in the APC, TP53, and KRAS genes along with BAT26 microsatellite instability via the modified solid-phase mini-sequencing method and reported both very high sensitivity (91%) and specificity (93%) for CRC diagnosis (69, 70). It is worth noting that all data collected from Ahlquist's survey also affirmed the accuracy of such alternative molecular tests for colorectal neoplasia screening (CRC and adenomas) compared with the current FOBT assay (71).

Given previous preclinical case-control studies, modern multi-biomarker fecal DNA assays, particularly regarding methylated indicators (NDRG4, BMP3, TFPI2, Vimentin tested by the Quantitative allele-specific real-time target and signal amplification (QuARTS) assay), mutation markers (KRAS mutations tested by direct sequencing), and an evaluation of fecal hemoglobin (Hemo Quant test), have been demonstrated to diagnose CRC and advanced adenomas at high sensitivity (85% and 60%, respectively) (69). Similarly, a Controlled clinical trial study also reported a high diagnostic precision of DNA analysis for the early detection of colorectal tumors (92.3%) and progressed premalignant lesions (42.4%) (14). Furthermore, the study of the size and purity of L-DNA fractions (oligonucleotide-based hybrid captures) and individual Alu sequences (qPCR technique) besides KRAS mutational analysis (Droplet Digital PCR (ddPCR) and single-strand conformational polymorphism (SSCP) methods) in stool samples indicated these measures as supplemental non-invasive approaches to routine screening strategies for CRC (72-74) (Table 2).

mRNA Indicators of CRC in Stool

The utility of discerning stool messenger RNAs (mRNAs) for CRC checkups has already been displayed, although mRNAs are deemed relatively unstable in stools compared with DNA, proteins, and microRNAs. Several trials have declared the uncovering of assorted CRC-specific mRNA, namely, MYBL2, MMP7TP53, and PTGS2, at elevated amounts in specimens of cases with CRC compared with the specimens of normal participants. Despite that, different sensitivity values were reported for MMP7 (31–65%) and PTGS2 (34–90%) based on the mRNA origin, extraction assay, and PCR calibration settings. The overrepresentation of ITGA2, PTGS2, ITGA6, and CEACAM5 in the multitarget fecal mRNA screening of AA subjects and the risk of degradation of fecal mRNAs must also be considered when using mRNA targets (62, 90) (Table 2).

Epigenetic Markers of CRC in Stool

Promoter Methylation of Stool DNA

Genetic and epigenetic alterations come together in a complex set of pathological molecular conductors in colorectal neoplasia formation, representing the molecular steps of adenoma-carcinoma transition (91). In particular, one of the fundamental epigenetic processes associated with colorectal neoplasia pathogenesis is the elevated CpG islands methylation of specific promoter regions that dysregulates cellular growth, division, and apoptosis (92). Therefore, evaluating such epigenetic variations at the DNA

Table 2: Review of approaches to detecting DNA and RNA genetic and epigenetic biomarkers in feces samples for colorectal cancer screening and diagnosis.

Biomarker	Approach	Assay	Sensitivity	Specificity	Reference
APC, TP53, KRAS genes and BAT26	Mutation & microsatellite instability testing	Modified solid-phase mini sequencing, PCR	91%	93%	(69)
NDRG4, BMP3, TFPI2, MGMT, hMLH1, vimentin, and KRAS	Methylation and mutation testing	QuARTS and direct sequencing	75-85%	84-92%	(69)
Fecal DNA integrity	L-DNA fractions	Oligonucleotide-based hybrid captures	52- 56.2%	92-96.3%	(73)
KRAS	Mutation testing	ddPCR and SSCP	*NA	* NA	(72)
Alu	Repetitive DNA sequences	qPCR	44%	NA	(75)
SFRP1	Methylation testing	PCR	52%	92%	(76)
ATM, MGMT, hMLH1, APC and HLTF	Methylation testing	PCR	75%	90%	(77)
GATA4/5,NDRG4 Vimentin, *SFRP2*	Methylation testing	PCR	96.4%	82- 95%	(78)
SDC2	Methylation testing	LTE-qMSP	77.4–81.1%	88.2–98%	(79)
BMP3 and VAV3	Methylation testing	QuARTS	92%	90%	(80)
RASSF2 and SFRP2	Methylation testing	PCR	79%	93%	(79)
GSTP1, MGMT, APC, SFRP2, HLTF, ATM, and hMLH-1	Methylation testing	PCR	38- 89%	55% -100%	(81)
MMP7, COX-2	Expression level analyzing	RT-PCR	87%	65%	(82)
MYBL2, MMP7, TP53 and PTGS2	Expression level analyzing	real-time PCR	58.3%	88.1%	(83)
ITGA2, ITGA6, PTGS2, CEACAM5	Expression level analyzing	real-time PCR	89%	95%	(84)
ITGA6	Expression level analyzing	ddPCR, real-time PCR	*NA	96%	(85)
miR-199a-3p, miR- 134, miR-17, miR-196a, miR-96, miR-7, miR-21, miR-92a, miR-106a, miR-183, miR-20a, miR214, miR-203, miR-326, miR-16, miR-125b, miR-126, miR-320, miR-145, miR-146, miR-320, miR-484-5p, miR-146a, miR-29b, miR-127-5Pa, miR-938, miR-9, miR-122 , miR-138 miR-135b miR-223 and miR-144, miR20a-5p, miR21-3p, and miR141	Expression level analyzing	qRT-PCR	50%	80%	(75, 86-89)

APC: Adenomatous polyposis coli, KRAS: Kirsten rat sarcoma virus, NDRG4: NDRG Family Member 4, BMP3: Bone morphogenetic protein 3, TFPI2: Tissue factor pathway inhibitor 2, MGMT: Methylguanine methyltransferase, hMLH1:human mutL homolog 1, ddPCR: Droplet Digital PCR, SSCP: Single-strand conformational polymorphism, qPCR: quantitative polymerase chain reaction, SFRP1: Secreted Frizzled-related Protein 1, ATM: ataxia telangiectasia mutated, HLTF: Helicase-like transcription factor, GATA4: GATA Binding Protein 4, NDRG4: N-myc Downstream-Regulated Gene 4, SDC2: Syndecan 2, LTE-qMSP: linear target enrichment quantitative methylation-specific real-time PCR, RASSF2: Ras Association Domain Family Member 2, GSTP1: Glutathione Stransferase Pi, MMP7: Matrix Metallopeptidase 7, COX-2: Cyclooxygenase 2, mybl2: MYB Proto-Oncogene Like 2, PTGS2: Prostaglandin-Endoperoxide Synthase 2, ITGA2: Integrin alpha 2, CEACAM5: CEA Cell Adhesion Molecule 5, itga6: Integrin Subunit Alpha 6, NA: not available

level by repeated cfDNA extraction from colonocytes shed in stools may allow the accurate diagnosis and screening of CRC and adenoma lesions (93). The analytical sensitivity of a multimarker promoter methylation panel (MGMT, hMLH1, and vimentin) of fecal DNA tested by Sure methylation-specific PCR or MS-PCR was reported at 75.0% for CRC vs. 59.6% for colorectal adenomas. The high specificity of stool methylated *SFRP1* gene was also reported (76). The frequent epigenetic promoter hypermethylation of multiple major genes, including ATM, MGMT, hMLH1, APC, and HLTF, varied from 45% to 55% (86), besides the confident high sensitivity and specificity of methylated vimentin (MBD capture and

MSP assays) (80, 94), GATA4/5and NDRG4 (MSP assay), as well as methylated SDC2 biosignatures (linear target enrichment quantitative methylation-specific real-time PCR (LTE-qMSP)) was approved in a different approach to diagnosing CRC early. It clearly was defined that the abnormal methylation of exon-1 sequences across the unexpressed vimentin gene (MS-PCR), BMP3, and VAV3 methylation (multiplex quantitative allele-specific real-time target and signal amplification) (78, 95-98) with 90% specificity can be reliable for CRC diagnosis and surveillance. Several main lines of data highlighted the practical importance of RASSF2 and SFRP2 promoter methylation of sDNA for clinical screening of both precancerous adenoma and sporadic/familial forms of CRC as well as for gastric cancer monitoring. Also, a panel of seven CRC-associated genes (GSTP1, MGMT, APC, SFRP2, HLTF, ATM, and hMLH-1) was evaluated at supporting the high sensitivity of aberrant methylated sDNA testing compared to COX-2 mRNA analysis (reverse transcription polymerase chain reaction (RT-PCR)) in CRC detection. The constant and trustworthy outcomes of the methylation-sensitive high-resolution melting (MS-HRM) assay combined with emulsion PCR (emPCR) with hydrogel immobilized bead-array technique showed a great sensitivity and specificity for both sDNA-based SFRP2 and VIM gene methylation levels for screening of CRC (77, 79, 81, 82, 99) (Table 2).

miRNAs Markers of CRC in Stool

miRNAs are intricate in the pathogenesis of varied types and subtypes of malignancies, particularly CRC. The opportunity of employing circulating or stool miRNAs as non-aggressive biomarkers unlocks interesting prospects for their practical clinical applications. Moreover, it has been proposed that the primary discernable tumor cells and/or tumor-induced molecule alterations of CRC can be detected in stool samples considerably more than in the bloodstream, signifying that stools are suitable specimens for early detection and screening approaches. A prior study announced that exosomes or cell-mediating coverings shield miRNAs from being broken down by RNAses, even in stool (100-102). Above all, the market accessibility of potent high-efficiency procedures for comprehensive miRNA appraisal, like microarrays and easier, commonly convenient quantification methods to miRNA profiling, namely quantitative real-time reverse transcription PCR(qRT-PCR) (preferably for assessing minor miRNA panels) and NGS, which usually distinguishes miRNA variants, could be very valuable for each miRNA analysis (103). According to several detection rates of each miRNA, the entire sensitivity was 50%, and the specificity was 80% for diverse diagnostic miRNAs in CRC. Previous RT-qPCR data indicate that multiple miRNAs, including miR-199a-3p, miR-134, miR-17, miR-196a, miR-96, miR-7, miR-21, miR-92a, miR-106a, miR-183, miR-20a, miR214, miR-203, and miR-326 had stool-elevated expression compared with healthy controls for CRC according to the Dukes' and TNM carcinoma stages (104, 105). Conversely, decreased expression was assessed for signature miRNAs including miR-16, miR-125b, miR-126, miR-320, miR-145, miR-146, miR-320, and miR-484-5p in fecal specimens from patients with diagnosed CRC in higher stages (TNM III-IV). Also, the reduced expression of other stool microRNAs like miR-146a, miR-29b, miR-127-5Pa, miR-938, miR-9, miR-122, and miR-138 (real-time PCR) have been seen from initial to latest TNM stages of patients with colorectal malignancy. Fecal-based miR-135b can be utilized as the best marker for the recognition of CRC and progressed adenoma (86, 87, 105, 106). The practice of a two-miRNA detection panel by miR-223 and miR-144 (qRT-PCR) showed respectable sensitivity and equitable specificity for the promotion of a non-invasive follow up method for colorectal carcinomas. Furthermore, findings from another study validated the usefulness of a large-scale study regarding determining the important patterns of fecal miR20a-5p, miR21-3p, and miR141 (qRT-PCR) in the first and second screening of CRC after follow-ups after the therapeutic operation (75, 88, 89) (Table 2).

Fecal DNA and RNA-based Procedures in CRC Screening, Diagnostics, and Prognosis

Colorectal cancer results from an aggregation of genetic and epigenetic instabilities genome-wide, especially variations in DNA methylation. Accordingly, specific DNA deviations that are abnormally methylated in colorectal malignancies are well-established as the best non-invasive molecular indicators for early CRC diagnosis (107). Despite its precision, colonoscopy is uncomfortable, invasive, and requires gut preparation. Thus, a non-invasive and perfect new screening method such as the EarlyTect™ displayed good specificity and sensitivity for SDC2 methylation discovery in favor of a non-invasive screening procedure for early diagnosis with strong accuracy. SDC2 methylation had identical sensitivity for screening CRC as the mt-sDNA diagnostic assay (Cologuard), which estimates the existence of methylated TFP12, BMP3, NDRG4, and VIM genes, along with mutant KRAS and fecal hemoglobin. The sensitivity (50–92%) and specificity (80–100%) of SDC2 methylation for CRC diagnosis are reportedly high (39, 108, 109). Abnormal SDC2 methylation repeatedly appears in the earliest cancerous neoplasms, is retained in progressed colorectal cancer, and is not affected by factors like age, sex, race, and disease stage. Zhang et al. expressed sensitivity and specificity of 73% and 92% for CRC and 51% and 92% for adenoma about the merged individual- and complex-gene methylation testing of fecal DNA specimens (110). These findings' reliability is debatable, so efforts

to unite numerous methylated genes in panels have been taken to enhance test reliability. However, in earlier research, the bearing of mutations in stool DNA was stated to increase novel non-invasive procedures for CRC initial diagnosis and screening. Supplemental monitoring assayed objective genes (p53, APC, PIK3CA, and K-ras gene mutations, long DNA trial, and microsatellite instability indicator BAT-26 deletions) in a considerably sensitive and numerical pathway (66, 111, 112). Consequently, the mean frequency of mutant DNA in fecal specimens did not diverge significantly through various stages of CRC (I, II, III, and IV), reported as 0.83%, 0.31%, 0.20%, and 0.62%, respectively (113).

It is recently recognize that signature assays, including multimarket fecal testing, will introduce as the best only when several biomarkers are analyzed together. The first study of this assay involving methylation of TFPI2, NDRG4, BMP3, and VIM genes, K-ras mutation, and DNA assessment with β-actin evaluation and the HemoQuant assay for hemoglobin reached indicative sensitivity of 78% to 85% and specificity of 85% to 90% in case-control research (114, 115). It is extraordinary that this assay performed substantially superior in relation to the examination for SEPT9 DNA methylation in plasma (analogous to Epi proColon). Screening utilization of this investigation in a broad survey showed CRC recognition sensitivity of 92.3% at a specificity of 86.6%, among other diagnostic DNA biomarkers (116, 117). Specimens of blood, feces, intestinal lavage fluid, and colorectal mucus were further checked for whole and ALU-situated DNA measurement, DNA accuracy estimation, investigation of coding-gene expression, and long non-coding RNA (25). Long DNA was additionally assessed by testing human Alu duplicates, revealing a sensitivity of 44% and specificity of 100%. Long DNA ratios have been more quantified alongside iFOBT, displaying that a mixed strategy improved the prediction of cancer or high-risk colonic adenoma precursors (118). None of these methods might supply acceptably great counts for a sensitive and specific screening. Cao et al. reported a fecal-derived methylated C9orf50 assay sensitivity of 95.0% and a specificity of 85.9% for detecting precancerous lesions and all-stage CRCs. The stool-based methylated KCNQ5 test also demonstrated a high sensitivity for diagnosing premalignant injuries and initial phase colorectal tumors and a sensitivity of 77.3% for advanced phase colorectal tumors—noticeably less than that of methylated C9orf50 (119). Larger amounts of them are affiliated with the carriage of CRCs, implying that microRNA quantification in feces or blood specimens could furnish a novel indicatory style for CRC in the initial diagnosis and follow-up (120). Multiple released data from fecal specimen testing emphasized miR-21 importance as a main diagnostic biomarker ,while not exhibiting remarkable sensitivity and specificity ratios. MiR-223 and MiR-451 observable in feces made a higher sensitivity and specificity in a limited survey (121). Other researches explored supplemental microRNAs including miR-106, miR-20b-5p, miR-144 miR-135b, miR-221, miR-17-93, miR-92, miR-20 group and miR-18 as strong CRC biosignatures (86, 122). Although none of these miRNAs demonstrated sufficient prognostic quality for utilization in favor of a single CRC analytical exam, subsequent considerations are needed to improve the analytical quality of miRNA by explaining tumor-linked alterations of RNA status in fecal specimens (122).

An anomalous expression of CD44 has been registered in the stools of 60 to 70% of colorectal subjects before surgery but in merely 10 to 30% of subjects following surgery. Measurement of cyclooxygenase 2 (COX-2) (123) mRNA exclusively or in association with MMP-7 mRNA has been evaluated for CRC diagnosis ; however, other researchers found COX-2 mRNA in just 50% of tumor cases (124, 125). A 2011 study found that cases with elevated stool KIAA0247 mRNA amounts had a meaningfully increased five-year overall survival rate, possibly related to the therapeutic benefit of the 5-FU regime (126).

Different data demonstrated that *SFRP1* and *SFRP2* methylation assays, as non-invasive modalities, have promising accuracy for detecting CRC and its early development stages. Besides, *NDRG4* and *VIM* could also be considered significant diagnostic marker genes in CRC and adenoma, respectively. Nonetheless, it is pretty undecidable if these findings, and those of various further examinations, reflect true dissimilarities in mRNA quantities or are technical artifacts produced through specimen reading, derivative RNA durability, or restriction of either the PCR reactions or the reverse transcription (RT) (127, 128) (Figure 1).

Concluding Recommendations

Molecular indicators that might be applied to follow up or prognosticate a recurrence in a preclinical step of screening could have a major effect on the direction and, feasibly, the survivability of colorectal cancer cases. Multiple researchers have suggested using stool nucleic acid assessments as a method for CRC screening. The isolation of DNA and RNA from stool is an uncomplicated, comparatively noninvasive, and inexpensive method, that result in genetic and epigenetic alterations detection, although examination of gene changes is usually costly and time-consuming. Indeed, molecular modifications in malignant and primary neoplasms gathered from patients can alter, increasing aggressiveness and/or sensitivity to medications. Therefore, the potential of molecular surveillance changes through ordinary trials relies on nucleic acids extracted from feces specimens to be able to authorize a highly applicable evaluation of how behaviour of disease and theraupathis decisions. The findings from research done in this field further stress dramatic variation in

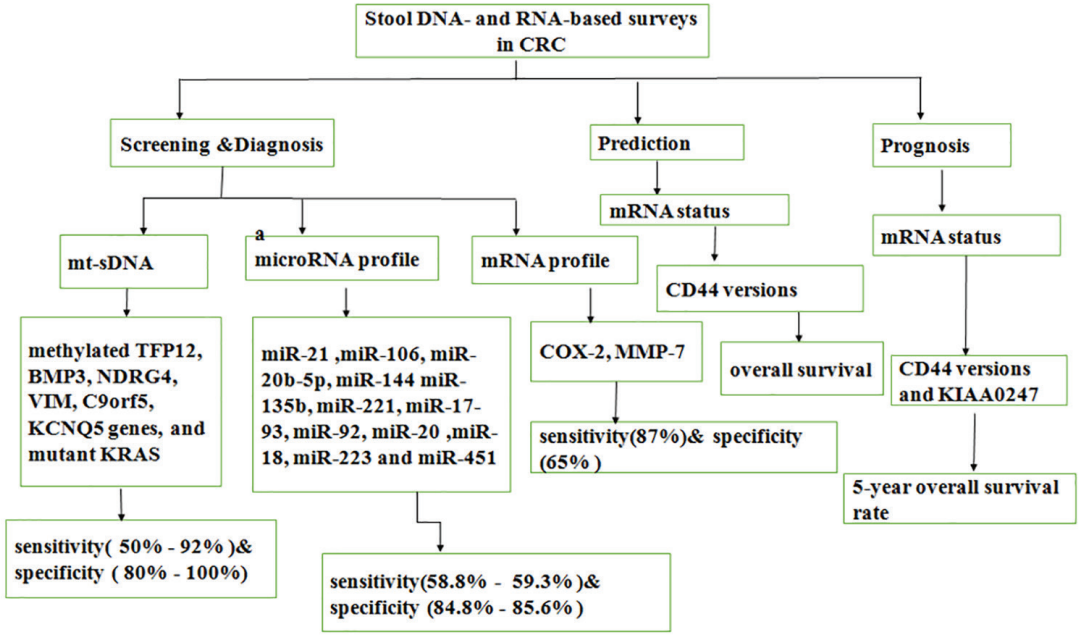

Figure 1: A schematic summary of the prevalent and powerful Stool DNA- and RNA-based biomarkers in CRC. MT-sDNA: Multi-target stool DNA, TFPI2: Tissue factor pathway inhibitor 2, BMP3: Bone Morphogenetic Protein 3, NDRG4: NDRG Family Member 4, VIM: Vimentin, C9orf50: Chromosome 9 Open Reading Frame 50, KCNQ5: Potassium Voltage-Gated Channel Subfamily Q Member 5, COX-2: Cyclooxygenase 2, MMP7: Matrix Metallopeptidase 7, DRAGO (KIAA0247): drug-activated gene overexpressed

respect of DNA and RNA amount, output, specificity, and sensitivity, implying the existence of several pre-analytic (intricacy of specimen management, the presence of dynamic nucleases, the output in human genomic DNA, human DNA integrity, and existence of PCR blockers) and methodic items (high qualified fecal DNA quantification through spermidine adding, use of magnetic bead-based fecal miRNA Sequence via stem-loop RT and locked nucleic acid (LNA) primers), which would improve the diagnostic quality of the technique.

Furthermore, human stool RNA is a little-studied kind of biosignature because of the problem of specimen maintenance. Specimen gathering, storage, and processing are vitally significant considerations for DNA and RNA isolated from feces as they affect the related assays. Consequently, standardization of specimen gathering and evaluation is essential to ensure the accuracy of diagnosis or prognosis, and large, multi-center studies are needed to delineate the exact role of these molecular indicators in medical practice.

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Author Contribution

P.M. and S.I. conducted the conceptualization and design. S.I., P.M., M.Z., F.Sh., M.S., H.A., H.B., and M.J.L. contributed to data collection, analysis, and interpretation. S.I. wrote the first draft of the manuscript. P.M., M.Z., H.A., H.B., and M.J.L. revised the manuscript. All authors have read and approved the final version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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