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Molecular Biomarkers of Colorectal Cancer and Cancer Disparities: Current Status and Perspective

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Abstract

This review provides updates on the efforts for the development of prognostic and predictive markers in colorectal cancer based on the race/ethnicity of patients. Since the clinical consequences of genetic and molecular alterations differ with patient race and ethnicity, the usefulness of these molecular alterations as biomarkers needs to be evaluated in different racial/ethnic groups. To accomplish personalized patient care, a combined analysis of multiple molecular alterations in DNA, RNA, microRNAs (miRNAs), metabolites, and proteins in a single test is

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Conflict of Interest

Uponder Manne, Trafina Jadhav, Balananda-Dhurjati Kumar Putcha, Temesgen Samuel, Shivani Soni, Chandrakumar Shanmugam, and Esther A. Suswam declare that they have no conflict of interest.

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required to assess disease status in a precise way. Therefore, a special emphasis is placed on issues related to utility of recently identified genetic and molecular alterations in genes, miRNAs, and various “-omes” (e.g., proteomes, kinomes, metabolomes, exomes, methylomes) as candidate molecular markers to determine cancer progression (disease recurrence/relapse and metastasis) and to assess the efficacy of therapy in colorectal cancer in relation to patient race and ethnicity. This review will be useful for oncologists, pathologists, and basic and translational researchers.

Keywords

Colorectal cancer; Biomarkers; Exosome; Metabolome; Kinome; Genetics; Epigenetics; Tumor markers; Disparity

Introduction

Background on the Disparities of Colorectal Cancer (CRC)

Advances in strategies for the development of cancer biomarkers, increasing our understanding of cancer pathobiology, have resulted in identification of several candidate molecular biomarkers. Most of these candidates, however, fail to reach the clinic because the causes of tumor development, rates of progression, and responses to therapeutic interventions can be unique to individual patients. Furthermore, various factors, including cellular and molecular heterogeneity within tumors, varying host immune responses, diet, environmental exposure, lifestyle, and patient demographics, influence the effectiveness of cancer biomarkers. Thus, the *one-size-fits-all* approach is obsolete, and there is a need to design strategies to develop effective biomarkers by applying principles of individualized cancer care.

CRC is more common and more aggressive among African American (AA) patients [1, 2]. Furthermore, as determined in our and other studies, molecular determinants governing tumor initiation and progression are distinct in AA and Caucasian patients [3–7]. Differences in tumor biology may contribute to the more aggressive CRC in AA patients [5, 8]. Genes mutated or differentially expressed among these populations are involved in cellular functions that promote tumor growth and metastasis. Metastasis-associated genes are preferentially overexpressed in tumors of AA patients [8]. Different incidences of microsatellite instability (MSI) and levels of methylation for relevant genes are possible factors in CRC racial disparities [3, 9]. Our group has found genetic differences and noted an association between a p53 variant and poor cancer-specific survival of AA patients [5]. Further, AA patients with increased expression of microRNA-181b have worse survival [10]. The molecular determinants contributing to the racial/ethnic disparity of CRC remain to be elucidated. This review highlights the molecular basis for CRC in relation to patient race/ethnicity.

Cancer Biomarkers and Biomarker Types

A tumor marker, or *cancer biomarker*, is produced either by a tumor or by the host in response to the tumor. The physiological expression of the presence of a tumor is a characteristic that is objectively measured and evaluated as an indicator of normal biologic

processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. These indicators can be measured by genetic, proteomic, cellular, or molecular substances found in higher than normal amounts in the blood, urine, or body tissues of a cancer patient. Moreover, a biomarker must be measurable, reproducible, and linked to relevant clinical outcomes and must demonstrate clinical utility. Biomarkers are measurable and reliable indicators, categorized by utility, that are used to assess the disease process or outcome or to estimate treatment efficacy (see Table 1) [11].

In recent years, a relatively new concept, the development of *companion diagnostics*, is emerging to move the field of *personalized medicine*. Typically, companion diagnostic tests are developed separately and are designed to be paired with an existing therapeutic agent, a diagnostic assay, or both to improve the efficacy. The companion test can be an in vitro diagnostic device or an imaging tool. Additionally, companion molecular markers aid in stratifying patients into subsets with distinct clinical outcomes [12].

The basic/translational research, medical, and pharmaceutical communities recognize the advantages of biomarkers. By replacing clinical endpoints, biomarkers can reduce time and costs for phase I and II clinical trials. They can also be helpful in redefining diseases and advancing therapies by shifting the emphasis from traditional practices depending on symptoms and morphology to more rational, objective, and evidence/molecular-based therapies.

Biomarkers of CRC

Biomarkers and Cancer Disparities

In regard to CRC, findings of earlier efforts, focused on identifying molecular bases for racial/ethnic disparities, are inconclusive and not clinically useful due to various confounding factors, including the effect of population admixture and the molecular/genetic heterogeneity of these cancers. Investigations performed by us and others show differing biologic consequences based on pathologic stage, tumor location, and the race/ethnicity of CRC patients. Thus, there is a need to identify and validate biomarkers for CRC by the race/ethnicity of patients. Such studies would need to be accomplished with large tissue cohorts of CRC collected from different racial/ethnic groups (e.g., AAs, Caucasians, and Latinos) of rural–urban and other geographical regions (e.g., northern–southern populations of the USA). The tissues and patient populations should be characterized for histologic, pathologic, clinical, socioeconomic, behavioral, exposure, and epidemiologic features. Such biomarker studies may establish the molecular basis linking biologically based differences in patient demographic (age/race/ethnicity/gender) groups to cancer outcomes. Further, the data may provide a rationale for evaluation of population-based biomarkers in the *personalization of cancer therapies*.

MicroRNAs as a Source of CRC Biomarkers and Health Disparity Markers—

MicroRNAs (miRNAs) are non-coding RNAs of ~17 to 27 nucleotides that regulate gene expression at the post-transcriptional level through interaction with the 3′-untranslated regions (3′ UTRs) of the target messenger RNAs (mRNAs). Expression patterns of miRNAs vary, depending on the tissues, and also differ between normal and cancer tissues. The small

size and stem-loop structure of miRNAs render these molecules more resistant to degradation by RNAases than mRNAs. They are thus likely to be conserved and easy to detect in archival tissues and in body fluids such as blood, urine, and saliva [13]. The stability of miRNAs in feces, serum, plasma, and urine allows them to be acquired by minimally invasive procedures, and they can serve as sensitive biomarkers [14]. The stability of miRNAs in these sources might be due to their encapsulation in extracellular membrane-enclosed vesicles or exosomes (see below).

Platforms to study miRNA expression patterns have evolved from northern blots, in situ hybridization, real-time PCR, and primer extension to increasing use of microarrays and high-throughput sequencing to profile the genome of miRNAs [15]. The differential expression of miRNAs correlates with cancer type, stage, and other clinical variables, thereby making miRNA profiling a tool for cancer diagnosis and prognosis [16]. miRNA expression signatures can serve as prognostic cancer biomarkers [17].

The first reported association of miRNA deregulation in CRC tissues was in 2003 [18]. Aberrantly expressed miRNAs contribute to the development and progression of cancers by targeting tumor suppressors or oncogenes [19]. Upregulation of miR-135, which targets and reduces levels of the adenomatous polyposis coli protein, is an early event in CRC and is a potential biomarker for the early detection of CRC [20]. Expression levels of miR-92a can be used to distinguish CRC from healthy controls with 83.0 % sensitivity and 84.7 % specificity [21, 22].

The prognostic value of single miRNAs and large-scale miRNA panels has been assessed [23], and their high expression in CRC tissues has been reported [23,24]. Our studies of CRC have established that high expression of miR-21 is associated with a poor prognosis for patients, especially for non-Hispanic Caucasians [4]. In patients with CRC, serum levels of miR-21 increase with the stage of the disease and are associated with poor patient survival [25, 26]. Furthermore, there are reductions in serum miR-21 levels after surgical removal of CRC [25, 27]. Moreover, miRNA expression profiles are different in CRC patients with MSI relative to those with microsatellite stability (MSS) [28]. This observation has implications in CRC prognosis and decisions regarding treatment of patients, as those with MSI have a favorable prognosis relative to those with MSS.

To evaluate racial differences, a comparison of expression profiles of the whole human genome miRNA in colon cancer tissues collected from AA and Caucasian patients was made. The results show that expression of miR-182, miR-152, miR-204, miR-222, and miR-202 correlates with race. The twofold upregulation of miR-182 in tumors of AAs in relation to Caucasians is linked to reduced expression of downstream targets, FOXO1 and FOXO3A [29]. Similar race-dependent differences in expression levels of miR-182 are found in CRC, and elevated levels contribute to decreased survival of AA CRC patients [30]. In our race-based studies focused on miRNA expression in CRC, increased miR-21 expression correlated with a poor prognosis for Caucasian patients with stage IV CRC, but high miR-181b expression correlated with poor survival of only AA patients with stage III CRC [23]. Another study, conducted with Spanish patients with stage III CRC, showed that 11 miRNAs (miR-135b, miR-141, miR-18a, miR-20a, miR-21, miR-224, miR-29a, miR-31,

miR-34a, miR-92a, and miR-96) were overexpressed in tumors relative to their matching normal samples. Of these, only levels of miR-18a and miR-29a were higher in the sera of CRC patients [31]. In a proof-of-principle study, 12 miRNAs (miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106a, miR-134, miR-183, miR-196a, miR-199a-3p, and miR-214) had higher expression in the stools of CRC patients, and there were increased expression profiles for patients with late-stage disease. In contrast, a different study found low levels of a set of eight miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222, and miR-938) in the stools of patients with advanced stages of CRC [32]. In a Chinese cohort, low expression of miR-16 was a prognostic indicator of low 5-year survival relative to those with high miR-16 [33]. An additional study of Chinese CRC patients showed a correlation between high plasma levels of miR-221 and poor survival [34]. Overall, results of these population-based studies suggest that expression profiles of CRC are different in different racial/ethnic groups.

In addition to differential levels/expression patterns of miRNAs, single nucleotide polymorphisms (SNPs) within the miRNA genes can have effects on their functions and lead to an increased risk of cancer [35]. In a meta-analysis stratified by cancer type, there was an association of an *miR-196a2* polymorphism (rs11614913) with a risk of CRC [36]. For a Chinese population, this polymorphism may contribute to CRC susceptibility. In a case-control Chinese cohort, SNPs at *miR-196a2* (rs11614913) and *miR-146a* (rs2910164) were associated with a risk of CRC [37]. These results suggest that miR-196a2 is a universal biomarker for CRC risk. In a case-control study with Iranian patients, there was an association between rs1131445 SNP at the 3'UTR of the *IL-16* gene and the risk of CRC [38]. A meta-analysis showed that SNPs in mir-146a (rs2910164) and mir-149 (rs2292832) may increase the risk of CRC. As determined with pooled sample sets, the mir-196a2 (rs11614913) polymorphism may reduce the risk of CRC. Further, mir-499 (rs3746444) may relate to a decreased risk of CRC in Caucasians [39].

Since miRNAs modulate drug targets directly or indirectly, they have emerged as potential predictive biomarkers for CRC. Expression of miRNAs is altered upon treatment of CRC cell lines with 5-fluorouracil (5-FU). 5-FU reduces miR-200b, which lowers the levels of the protein tyrosine phosphatase, PTPN12, which, in turn, downregulates oncogenes, including *c-ABL* and *RAS*, resulting in decreased cell proliferation [40]. Further, altered miRNA levels can modulate the efficacy of biological and cytotoxic agents. *KRAS* mutations reduce the efficacy of anti-EGFR treatment. However, patients who are exhibiting *KRAS* mutations and high let-7 have an increased survival benefit from anti-EGFR therapy, for let-7 targeting *KRAS* and reducing its expression. miR-140 and miR-143 sensitize CRC cells to 5-FU [41, 42], and miR-192 downregulates the anticancer target, dihydrofolate reductase [43]. Additional mechanisms that alter response to therapy include SNPs in miRNA binding sites within the target mRNA. For patients with metastatic CRC, the LCS6 polymorphism in the let-7 binding site within the 3'UTR of *KRAS* predicts response to anti-EGFR-based therapy [44]. Similarly, SNPs in the 3'UTRs of base excision repair genes alter CRC prognosis and response to 5-FU [45].

The Proteome as a Source of CRC Biomarkers—The proteome is the set of proteins expressed by a cell, tissue, organ, or body at a certain (normal or pathologic) health

condition. The proteome or protein level/abundance represents the genomic status. Since mRNA transcripts resulting from different genetic alterations reliably predict variations in proteins in tumors, analyses of proteomes will help in phenotypic characterization of cancer patients. A mass spectrometry (MS) approach, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), is commonly used to discriminate tumor from normal tissue. Proteomic characterization of the genomically annotated TCGA colon cancers has indicated that protein abundance cannot be reliably predicted from levels of DNA or RNA; however, protein subtype signatures can be utilized for tumor classification [46]. The potential value of this approach to classify various clinicopathological features in CRC is illustrated by its correct prediction of disease recurrence, disease-free survival, and metastasis [47]. Fan and others (reviewed in [47]) employed a well-defined technology platform called ClinProt (Bruker Daltonics, Germany), based on magnetic bead purification of peptides, and MALDI-TOF-MS. They detected 61 short peptides, from 1 to 18 kDa, that were differentially expressed in the sera of patients with CRC, suggesting that this peptidome pattern may provide an alternative for CRC diagnosis or may help in tailoring the use of chemotherapy to each patient, the principles of which have applications to personalized medicine.

Additionally, MALDI-imaging MS (IMS), commonly known as MALDI imaging, has emerged as a useful tool for the molecular classification of tissue samples by disease stage, risk stratification, and therapy response and for the identification of disease biomarkers. For CRC, most proteomic studies have focused on the use of serum proteins as a diagnostic tool [47]. Most of these serum proteins, however, have not been evaluated for their prognostic potential and have not been validated with respect to racial disparities.

Genetic and Epigenetic Alterations as Biomarkers—Genetic abnormalities, including chromosomal and gene alterations and dysregulation of gene and protein expression, provide useful information for optimal care and outcomes of CRC patients. Chromosomal instability (CIN) is frequently observed in CRC, and loss of chromosome 18q is associated with a poor prognosis for CRC patients [48, 49]. MSI occurs in about 15–20 % of sporadic CRC cases and in >95 % of patients with the Lynch syndrome [50].

Comparison of CIN and MSI between AA and Caucasian CRC patients indicates that aberrations at chromosomes 11, 17p, and X are prominent in AAs. Aberrations in the genes, *THRB*, *RAF1*, *LPL*, *DCC*, *XIST*, *PCNT*, and *STS*, as well as the genes that are located on the 20q12-q13 loci (*TPD521.2*, *TOPI*, and *TNFRSF6B*) are distinct in AAs compared to Caucasians [51, 52], but their clinical values in CRC are yet to be determined.

Germline or somatic mutations in the *P53* gene are present in several human cancers, including CRC. These mutations are associated with aggressive tumor progression, chemoresistance, and a poor prognosis. Among the five exonic polymorphisms in *P53*, codon 72 polymorphism is well characterized for CRC progression and racial disparities [53–55]. Our group has shown that, although the incidences of all *P53* mutations are similar between AA and Caucasian CRC patients, the Pro/Pro phenotype of *P53* codon 72 polymorphism is present at a higher frequency in AAs compared to Caucasians, and its

higher frequency is associated with advanced tumor size and a poor prognosis for AA patients [5].

KRAS mutations are higher among AA patients (47 %) relative to Caucasian patients (43 %), especially in colon rather than rectal primary tumors, and may contribute to a poor prognosis [56]. *BRAF* mutations, the most common being V600E, occur in a small percentage of CRC tumors, but this mutation is associated with poor survival [57], suggesting the prognostic potential for the status of *BRAF* mutations. Differing rates of *BRAF* mutation are distinct in ancestral populations, with a lower frequency in Asian as compared to Caucasian and AA patients [58]. As determined in a population-based, case-control study of CRC in northern Israel, the prevalence of *BRAF*V600E is 5.0 %, and the mutation is more likely to be found in tumors from patients who are of Ashkenazi-Jewish descent [59]. Australians of Anglo-Celtic descents have a higher incidence of CRC and more *BRAF*V600E mutations than those of Southern European descent [60]. In a West African CRC patient population in Ghana, there is an absence of *BRAF* mutations [61].

Genomic instability at microsatellites (MSs) in tumors occurs due to the failure of the DNA mismatch repair (MMR) system to correct errors that are introduced during DNA replication. To determine the MS status, a panel of five or more MS markers is examined [62, 63]. Relative to non-Hispanic Caucasians, a threefold high MSI (MSI-H) (instability in 30 % of the examined microsatellites) was found for AA CRC patients [3]. However, CRC patients with high MSI have a favorable prognosis relative to non-MSI patients. Three studies, evaluating the MS status in CRC based on race/ethnicity, show MSI frequencies in AA patients from 7 to 48 % [3, 64, 65]. One of these population-based studies suggests that AA CRC patients exhibiting MSI-H, compared to Caucasian patients with MSI-H, do not show changes in CD8⁺ T cell infiltration, which may contribute to a higher mortality due to CRC in AAs compared to Caucasians [66]. However, a study of CRC in Ghana found a high incidence of MSI-H in the native African population compared to the European population [61]. In CRC, detection of MSI helps to determine the natural history of the disease and to identify patients who might benefit from surgery alone [67].

Most CRC patients that demonstrate a high MSI show a loss of expression of one or more of DNA MMR proteins (MLH1, MSH2, MSH6, and PMS2) [68] and indicate the presence of a germline mutation in MMR genes in 95 % of hereditary non-polyposis CRC (HNPCC) or Lynch syndrome cancers. However, in approximately 15 % of sporadic CRC cases, a lack of expression of MMR genes occurs due to MLH1 promoter hypermethylation [69]. The distribution of mutations in specific exons of *MLH1* and *MSH2* differs between Yellow (Mongolian) and White populations [70]. Additionally, certain mutations are found only in the Yellow race, suggesting the importance of patient race/ethnicity in screening mutations of these genes.

A systematic analysis of genetic markers in the evaluation of cancer risk in different ethnic backgrounds suggests that, although the biological association for cancer risk alleles is broadly consistent across various ethnic groups, a significant association between a variant and cancer risk is not reproduced for most of the markers, partly due to confounding

genomic architecture [71]. The L1307K mutation in the *APC* gene, found in 6 % of the Ashkenazi–Jewish population, may predispose to CRC [72, 73].

Predictive biomarkers aid in distinguishing patients who respond to cytotoxic and biological agents from those who are non-responders. For example, in patients with metastatic CRC, mutations in *KRAS* and *BRAF* serve as biomarkers for determining the efficacy of anti-EGFR therapies involving panitumumab or cetuximab [74–76]. Similarly, the *PIC3CA* mutation is a potential predictive biomarker for determining longer survival of CRC patients with regular use of aspirin after diagnosis [77]. Another potential predictive biomarker for CRC is high MSI, which may be due to a less aggressive nature of tumors or to a higher sensitivity of MSI-H patients to 5-FU, a standard chemotherapeutic agent for CRC.

Mutations in the *TP53* and *dihydropyrimidine dehydrogenase (DYPD)* genes decrease the therapeutic response to 5-FU [78–80]. Variations of the *uridine diphosphate-glucuronosyltransferase 1A (UGT1A1)* gene have a predictive value for side effects and for the efficacy of irinotecan [81, 82]. However, prospective trials are required to determine the clinical utility of these mutations in identifying patients who are likely to suffer serious side effects of 5-FU toxicity.

DNA methylation is an epigenetic alteration. Hypermethylation of the *MLH1* promoter in the sera of MSI-positive sporadic CRC patients is associated with a higher risk of death [83, 84]. A multicenter clinical trial of a screening test for CRC, relating to hypermethylation of plasma septin-9, shows that the test detects CRC in asymptomatic, average-risk individuals. However, this method may not be widely applicable due to its poor sensitivity [85].

Hypermethylation of the *DYPD* gene may provide a mechanism for severe 5-FU toxicity [86]. However, *DYPD* promoter methylation is not a strong prognostic factor for severe toxicity to 5-FU [87]. The racial/ethnic disparities in DNA methylation patterns indicate that, in different ethnic groups, molecular markers are involved in determining the susceptibility to diseases. Racial disparities in DNA methylation patterns in CRC correlate with patient survival [88].

The CpG island methylator phenotype (CIMP) is implicated in a poor prognosis for CRC patients [89]. However, different subgroups of CIMP also exhibit MSI and mutations in *BRAF*, *KRAS*, and *TP53* [90, 91]. Therefore, the association between the CIMP phenotype and CRC prognosis remains controversial. A highly sensitive methyl-BEAMing technology was developed for quantification of methylation in DNA samples [92]. For early-stage CRC, the sensitivity of this approach was fourfold higher than that for levels of carcinoembryonic antigen (CEA).

A meta-analysis has revealed a direct (dose–response) relationship between obesity or higher body mass index (BMI) and risk of CRC [93]. Obesity is a favorable independent prognostic factor for CRC survival [94]. A meta-analysis of genome-wide association studies found that, although most of the BMI loci are conserved across populations of European and African ancestry, the 6q16 locus shows an association with individuals of African ancestry [95]. In addition, Human Intestinal Tract Chip (HITChip) analyses show changes in bacterial profiles for AA patients with colon polyps relative to healthy AA individuals [96].

Immunophenotypic Expression Biomarkers of CRC—Nuclear accumulation of p53 (p53^{nac}), detected by immunohistochemistry (IHC), is a poor prognostic marker for proximal CRC in Caucasians, and high-grade CRC leads to shorter survival times for AAs than for Caucasians [97]. Other prognostic molecular markers that are independent of pathologic stage include phenotypic expression of Bcl-2, MUC-1 (mucin core protein), and p27kip-1, a cell cycle inhibitor. Oncogenes and tumor suppressor genes, such as *Bcl-2* and *P53*, are involved in the regulation of programmed cell death and cellular proliferation. Lack of Bcl-2 expression correlates with local invasion and metastasis. Several studies (reviewed in [98]) demonstrate that expression of Bcl-2 correlates with clinical outcomes primarily for stage II CRC and that it is a predictor for the recurrence and survival of CRC patients, especially those with stage II disease. A meta-analysis [98] confirms that Bcl-2 is a useful prognostic marker for CRC. Although increased expression of the core peptide of MUC-1 (a mucin antigen) is associated with a poor prognosis for CRC, phenotypic expression of MUC-1 is a prognostic factor for CRC of Caucasians but not of AAs [97]. No variation in the prognostic importance of MUC-1 was found based on the anatomic location of the tumors, a prognostic characteristic of CRC in AAs.

Meta-analyses indicate that overexpression of hypoxia-inducible factors (HIFs) is associated with increased overall survival (HR 2.06, 95 % CI 1.55–2.74) and disease-free survival (HR 2.84, 95 %, CI 1.87–4.31) of CRC patients [99]. Overexpression of HIF-1 α and HIF-2 α is associated with an unfavorable prognosis for CRC. However, HIF-1 α overexpression is associated with a worse prognosis for Asians relative to Europeans and other racial groups.

CEA, one of the first known tumor biomarkers, is widely used for CRC patients [100, 101]. Elevated CEA is detected in CRC tissues and in the sera or plasma of the patients. CEA levels increase with a stage of the disease [101]. Although CEA is used to detect the recurrence of CRC, the specificity of CEA as a biomarker is limited, as CEA levels are also elevated in patients with epithelial tumors of non-intestinal origin, patients with liver disease, and in smokers [102, 103].

Serum lactate dehydrogenase (LDH) has been used as a predictive marker for drug-induced hypersensitivity reactions in Japanese patients with advanced CRC [104]. Similarly, C-reactive protein (CRP), used as a marker for CRC, varies across racial/ethnic population groups [105–108].

The overall burden of germline MMR deficiency varies in different populations. The use of IHC staining of at least four MMR proteins is a useful screening strategy for diagnosis of the Lynch syndrome. Chew et al. [109] recommend routine screening of MMR deficiency for all young Asian CRC patients, in addition to the Amsterdam criteria, to detect Lynch syndrome.

Thymidylate synthase (TS) is used to predict lymph node metastasis of CRC in Chinese patients, and its overexpression correlates with lymph node metastasis, advanced tumor stage, increased 5-year recurrence rate, and decreased survival rate [110].

Molecular characterization of adenomatous polyposis coli (APC) and β -catenin proteins is often analyzed by IHC along with detection of mutations of *BRAF*, *KRAS*, *APC*, and *β -catenin* using direct sequencing. In a Chinese population, mutation of *APC*, rather than

BRAF(V600E), is the main cause for activation of Wnt signaling in right-colon-serrated polyps [111].

Other Molecular Assays for CRC—Fecal biomarkers for CRC include the fecal occult blood test and detection of mutations and hypermethylation of genes for fecal DNA shed from CRC cells. Several fecal DNA markers have been evaluated for CRC screening. For example, a simplified stool DNA test for hypermethylation of the vimentin gene and a two-site DNA integrity assay showed high sensitivity (83 %) and specificity (82 %) for CRC [112].

Multigene assays are likely to provide more robust information and serve as strong prognostic and predictive biomarkers. Several gene expression signatures have been developed to assess the prognosis for CRC patients [113–115]. A seven-gene expression prognostic test, ColoGuidePro, was developed for patients with stage II and III CRC [116]. Recurrence score assays, such as the Oncotype DX test™, which is based on gene expression profiling, were designed for determination of a risk of recurrence for stage II and III CRC and for breast and prostate cancer patients after surgery. Similarly, ColoPrint® is effective in determining a recurrence risk for stage II CRC patients and in identifying patients who can be safely managed without chemotherapy [117]. These assays have been validated with large cohorts of patients. Recently, the efficacy of the Oncotype DX test™ in AA and Caucasian stage II CRC patients was evaluated by us, and it was found that, after controlling for clinical and pathologic covariates, the means and distributions of recurrence score results and gene expression profiles showed no statistically significant difference between these two patient groups [118].

Currently, there is an effort to identify biomarkers that can be obtained with minimally invasive methods and persist beyond surgery. Two such approaches involve a study of circulating tumor cells and cell-free circulating tumor-associated DNA (ctDNA) in the blood of cancer patients. Several methods have been implemented to determine the quantitative and qualitative tumor-specific alterations of ctDNA, such as DNA strand integrity, gene amplification, gene mutation, gene methylation, and microsatellite abnormality as diagnostic, prognostic, and monitoring markers in cancer patients [119]. Although there are reports of their clinical use in CRC prognosis [120, 121], more work needs to be accomplished to optimize this application. However, comparisons of these biomarkers in the early diagnosis and prognosis in diverse ethnicities have not been made.

Prospective Molecular Markers for CRC

Exosomes as CRC Biomarkers

Exosomes are extrusions of cell membranes, measuring about 30–150 nm, and their contents are specific to normal and pathological conditions [122, 123]. These vesicles are involved in cell–cell communication; in autocrine, paracrine, and endocrine signaling; and in modulating immunity [124]. Exosomes are also produced by cancer cells, and the roles of their contents in cell transformation, angiogenesis, immune cell modulation, and metastasis have been evaluated [125–127]. Although exosomes are also released in non-pathological states, their contents, including miRNA, RNA, DNA, proteins, and lipids, may prove to be

useful, as they bear specific markers of their tissues of origin and are distributed through body fluids. Thus, exosomes are potential biomarkers for cancer [128]. In regard to CRC, exosomes carry cargoes that are specific to cells of gastrointestinal origin and can be utilized in establishing personalized therapies.

Currently, there is no regulatory agency-approved, exosome-based diagnostic, prognostic, or predictive clinical test for CRC. Nevertheless, a few molecular biomarkers that have a potential value in these areas are under investigation. Results with cultured cells and patient-derived samples suggest the exosomal presence of mir-18 [129] and a set of miRNAs that includes let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a [130]. The exosome profiles of miRNAs differ according to the *KRAS* status in that miR-10b is selectively increased in cells with wild-type *KRAS*, but only miR-100 is present in mutant-*KRAS* colon cancer cells [131]. Further, proteomic studies of exosomes from cancer cell lines have identified molecules that are yet to be validated as biomarkers in patient-derived samples. These candidates include molecules involved in metastasis (MET, S100A8, S100A9, TNC), signal transduction (EFNB2, JAG1, SRC, TNK1), and lipid raft biology (CAV1, FLOT1, FLOT2, PROM1) [132] and various immunomodulating molecules that act as autoantigens [133]. Furthermore, increased exosomal miR-19a in human serum samples correlates with the early recurrence of CRC [134]. Exosomes isolated from the ascite fluid of CRC patients contain hundreds of proteins [135], some of which may be developed as biomarkers for CRC in general or for a subset of CRC patients. Overall, the clinical potential for these and other exosome-associated molecules as biomarkers for CRC remains to be established. In sum, since exosomes can be obtained from patients non-invasively by collecting blood, urine, saliva, or other body fluids, these microvesicles hold a promising potential as sources of diagnostic, prognostic, and/or predictive biomarkers.

The Metabolome as a Source of CRC Biomarkers

The metabolome, defined as the set of metabolites of less than 2000 Da molecular mass present within a cell, tissue, or whole body [136], represents a unique source of biomarkers that reflect the physiological or pathological state at a given time. Metabolomics, downstream from gene expression, demonstrates complex biological changes and thus complements genomics, transcriptomics, and proteomics. These molecules are generally released into the microenvironment, in which the biological unit finds itself, and thus into the circulation at large. Since the metabolome reflects the phenotype of patients, the presence of metabolites in the circulation and the ease of collecting samples make it an attractive target for biomarker development.

With technological advances in methods to detect small molecules as well as increased high-throughput (NMR/MS-based analytical platforms) and bioinformatics capabilities to analyze thousands of metabolites simultaneously, metabolomics holds great promise to enhance the discovery of metabolites for diagnosis, prediction, and prognosis of a variety of cancers [137]. As a recent entrant into the field of cancer biology, metabolomics has not yet reached a stage for clinical trials or routine clinical use. Nevertheless, there is a potential for application of this powerful tool in the clinical oncology of CRC.

For CRC diagnosis, a prediction model based on MS analysis of the serum metabolome identified a set of metabolites, including 2-hydroxybutyrate, aspartic acid, kynurenine, and cystamine; its sensitivity, specificity, and accuracy were 85.0, 85.0, and 85.0 %, respectively. In contrast, the sensitivity, specificity, and accuracy of the CRC marker CEA were 35.0, 96.7, and 65.8 %, respectively, and those of the marker CA19-9 were 16.7, 100, and 58.3 %, respectively [138]. These results show that, although there is less specificity relative to CEA-based testing, the metabolomics set of biomarkers has superior values for sensitivity and accuracy. Moreover, the metabolite sets showed higher sensitivity for the detection of early-stage CRC. Of note, the serum metabolite composition may differ between localized and metastatic CRCs or even between liver-only metastasis and extrahepatic metastasis, opening the possibility of identifying patients at different stages of CRC [139].

In addition to liquid biopsies, solid-tissue biopsies can be used for metabolic profiling of CRC. MS analysis of tumor tissue identified a distinct type of metabolite profile associated with CRC relative to that of normal tissue and led to the identification of chemically diverse marker metabolites, which were associated with dysregulated biochemical processes, including glycolysis, TCA cycle, osmoregulation, steroid biosynthesis, eicosanoid biosynthesis, bile acid biosynthesis, and lipid, amino acid, and nucleotide metabolism [140]. Overall, the pool of metabolites found to be distinct between normal and CRC patients included (a) 3-hydroxybutyric acid, L-valine, L-threonine, 1-deoxyglucose, and glycine [141]; (b) a panel consisting of citrate, hippurate, *p*-cresol, 2-aminobutyrate, myristate, putrescine, and kynurenate [142]; (c) alanine, citrate, creatine, glutamine, peptide NHs, lactate, leucine, pyruvate, tyrosine, 3-hydroxybutyrate, acetate, formate, glycerol, lipid ($-\text{CH}_2\text{-OCOR}$), the N-acetyl signal of glycoproteins, phenylalanine, and proline [143]; (d) hydroxylated polyunsaturated ultra long-chain fatty acid metabolites [144]; (e) glucose, inositol, hypoxanthine, xanthine, uric acid, and deoxycholic acid [145]; (f) L-valine, 5-oxo-L-proline, 1-deoxyglucose, D-turanose, D-maltose, arachidonic acid, hexadecanoic acid, and L-tyrosine [146]; (g) proline, cysteine, acetate, and butyrate [147]; and (h) L-alanine, glucuronic lactone, and L-glutamine [148]. Most of these metabolites are yet to be validated but might serve as tools for the early detection, prediction, and prognosis of CRC [149–152].

Although metabolomics appears to be a powerful tool for clinical use in CRC oncology, substantial challenges need to be overcome. The most fundamental is the dynamic nature of metabolic processes and their susceptibility to a range of factors that are independent of CRC pathology but result from endogenous or exogenous factors. For example, although stools provide a direct, non-invasive source of the gastrointestinal metabolome [153], the inter-personal variation in gut microbiota, which has a substantial effect on the outcomes of the digestive process, could make it difficult to separate pathological changes from effects of the gut microenvironment. Indeed, a recent study shows that colonic microbiota alter the cancer metabolome [154]. Additionally, the effects of genetic background, time of day, health, nutritional status, and other spatial or temporal variations may pose challenges. Currently, population-specific metabolomic studies in CRC are few. A study of fresh fecal samples has demonstrated that changes in diet result in increased saccharolytic fermentation and butyrogenesis and suppression of bile acid synthesis in AAs who were fed a high-fiber and low-fat rural African-style food for 2 weeks [155]. Therefore, standardized protocols and combinations of biomarkers with high individual scores, in conjunction with appropriate

algorithms, are needed to establish biomarker platforms for CRC in general or for subgroup-oriented clinical use [156].

The Kinome as CRC Biomarkers

The kinome, defined as the protein kinase complement of the human genome, contains nine broad groups of genes [157]. The types of kinases are based on their capacity to phosphorylate various amino acids (e.g., serine and threonine or tyrosine, some both) and especially protein tyrosine kinases. These are regulators of intracellular signal transduction pathways, mediating development and multicellular communication [158]. In cells, including CRC cells, these kinases are tightly regulated in signaling pathways by various molecules, depending on the stimulus and cellular context [159, 160]. Since mutations in protein tyrosine kinases are rare in CRC, most research and clinical efforts that involve these kinases are geared towards targeting them for therapy rather than for biomarker applications.

Epidemiological studies have evaluated the risk for CRC associated with genetic variations in MAPK signaling pathways [161]. To determine if key driver CRC alterations occur at different frequencies in ancestral populations, 385 mutations across 33 known cancer genes in CRC DNA from 83 Asian, 149 AA, and 195 Caucasian patients were screened by the use of a high-throughput genotyping platform (OncoMap). Currently, the only candidate kinase considered for clinical CRC biomarker application is the serine–threonine kinase, BRAF [162].

Conclusions, Challenges, and Future Directions

Despite their impressive potential, several prognostic and predictive biomarkers are not in clinical practice due to various challenges. Failure in developing effective molecular cancer biomarkers is due, in part, to a lack of uniformity in implementation and interpretation of assay results, diversity of assay methods (platforms), heterogeneity in genetic alterations, admixture in study populations (patient race/ethnicities and demographics), and diversity in pathobiology of disease (geography-specific disease forms). Additionally, there is a lack of standardization of sample collection, processing, and stabilization techniques; standard specimen reference sets in specimen selection, collection, procession, and storage; statistical competence; and adequate clinical validation tools. Moreover, the field of cancer biomarker development is challenging because of a lack of understanding of the molecular pathogenesis of cancers and because advancements in developing new assay platforms are continuing to emerge.

Because biologically based differences in racial/ethnic and demographic (age/gender) groups have implications on clinical presentation and cancer outcomes [4, 6, 163–165], approaches to develop biomarkers should progress through population-based studies. Such biomarker studies provide a strong rationale and foundation for personalization of cancer therapies. To validate the clinical utility of molecular biomarkers, biomarker development studies need to be conducted in both academic and community settings, and emphasis should be placed on establishing collaborations with community oncologists to form tissue/patient data consortia to aid in performing studies on the development of cancer biomarkers. Such academic community-based efforts will (a) enhance translational cancer research for

rapid incorporation of molecular advancements and pivotal trial data into community practice and (b) facilitate the integration of biomarker profiles and epidemiological, nutritional, and behavioral research to have maximum impact of molecular biomarker testing and to help in providing personalized medicine.

Overall, the integration of biomarkers into routine clinical practice requires the efforts of experts from both academic cancer centers and community cancer caregivers. These experts include translational cancer biologists, pathologists, medical oncologists, surgical oncologists, gastroenterologists, community physicians, bioinformaticists, computational biologists, biostatisticians, epidemiologists, nutritionists, and behavioral scientists. Further, to address the moral, ethical, and social issues related to cancer biomarker development, there is a need to involve bioethicists and social scientists for their effective implementation.

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Table 1

Categorization of biomarkers based on clinical utility

Category	Clinical utility
Early detection	Used as a screening tool to find cancers, including early-stage neoplastic lesions
Diagnostic	Used to assess the presence or absence of cancer
Prognostic	Used to assess patient survival probabilities or to detect an aggressive phenotype and to determine how the cancer will behave (the natural history of cancer)
Predictive	Used to predict whether the drug and/or other therapies are effective or to assess the effectiveness of treatment
Target	Used to identify the molecular targets of novel therapies and to determine which molecular markers are affected by therapy
Surrogate endpoint	Used as a substitute for clinical endpoints to measure clinical benefit, harm, or lack of benefit or harm; can replace traditional endpoints, such as incidence of disease, mortality due to disease, or the recurrence or relapse of disease; or used for monitoring therapeutic intervention trials