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AIM AND SCOPE

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REVIEW

Biomarkers in colorectal cancer: Current clinical utility and future perspectives

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Abstract

Colorectal cancer (CRC) is a major cause of cancer death worldwide. CRC has poor prognosis and there is a crucial need for new diagnostic and prognostic biomarkers to avoid CRC-related deaths. CRC can be considered a sporadic disease in most cases (75%-80%), but it has been suggested that crosstalk between gene mutations (*i.e.,* mutations of *BRAF*, *KRAS*, and *p53* as well as microsatellite instability) and epigenetic alterations (*i.e.,* DNA methylation of CpG island promoter regions) could play a pivotal role in cancer development. A number of studies have focused on molecular testing to guide targeted and conventional treatments for patients with CRC, sometimes with contrasting results. Some of the most useful innovations in the management of CRC include the possibility to detect the absence of *KRAS*, *BRAF*, *NRAS* and *PIK3CA* gene mutations with the subsequent choice to administer targeted adjuvant therapy with anti-epidermal growth factor receptor antibodies. Moreover, CRC patients can benefit from tests for microsatellite instability and for the detection of loss of heterozygosity of chromosome 18q that can be helpful in guiding therapeutic decisions as regards the administration of 5-FU. The aim of this review was to summarize the most recent evidence on the possible use of genetic or epigenetic biomarkers for diagnosis, prognosis and response to therapy in CRC patients.

Key words: Biomarkers; Colorectal cancer; Epigenetics; Tumor markers; DNA methylation

Core tip: Colorectal cancer (CRC) is one of the leading causes of cancer death in the world today. Therefore, any improvement in early diagnosis, selection of appropriate treatment regimen, and effective follow up can be crucial in decreasing related mortalities.

This review discusses the most useful and promising genetic and epigenetic biomarkers for CRC. There is growing evidence that these biomarkers could help future development of more personalized treatment approaches.

Vacante M, Borzì AM, Basile F, Biondi A. Biomarkers in colorectal cancer: Current clinical utility and future perspectives. *World J Clin Cases* 2018; 6(15): 869-881 Available from: URL: http://www.wjgnet.com/2307-8960/full/v6/i15/869.htm DOI: http://dx.doi.org/10.12998/wjcc.v6.i15.869

INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer death worldwide, with an estimated 2.2 million new cases and 1.1 million deaths in the next ten years[1]. Therapeutic strategies for stage Ⅰ, Ⅱ, and Ⅲ disease includes surgery, adjuvant chemotherapy only for selected patients with stage Ⅱ and most patients with stage Ⅲ CRC, and radiotherapy for patients with stage Ⅱ and Ⅲ rectal cancers[2,3]. Palliative therapies are used for patients with metastasis or stage Ⅳ colorectal cancers that are not resectable; in these patients, the objective is to control symptoms and increase survival[4]. CRC has poor prognosis and there is a crucial need for new diagnostic and prognostic biomarkers to avoid CRC-related deaths[5]. Many recent studies have focused on molecular testing to guide targeted and conventional treatments for patients with CRC[6]. The molecular analysis of biomarkers for CRC is making great progress, but the inclusion of novel molecular tests into routine clinical practice faces huge challenges such as a better comprehension of genetic mutations in CRC, the need for laboratory techniques able to measure the resulting phenotypes and genotypes, and the achievement of regulatory qualification for clinical use. In 2011, a National Comprehensive Cancer Network (NCCN) Task Force aimed to assess the clinical utility of tumor markers for different cancer types (including CRC), and underlined common difficulties that clinicians may experience in the management of oncological patients, and then suggested recommendations for the community interested in developing tumor markers[6]. Many of the published findings on molecular biomarkers are controversial, and currently the most reliable molecular marker in clinical practice is the *KRAS* gene for patients receiving epidermal growth factor receptor (EGFR) - targeted therapy for CRC metastatic disease[7]. In 2017, an Expert Panel of The American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology, and American Society of Clinical Oncology developed guidelines that aimed to determine standard molecular biomarker testing of CRC tissues in order to direct EGFR therapies and standard chemotherapy regimens. The Expert Panel carried out a literature search that included more than 4000 scientific papers and concluded that mutations in EGFR signaling pathway genes may predict negative response to EGFR-directed therapies for CRC[8]. The process of carcinogenesis in CRC is related to different mechanisms that include, among others, chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and microsatellite instability (MSI)[9]. In 1990, Fearon and Vogelstein described a classical genetic model for colorectal cancerogenesis characterized by the accumulation of mutations in the adenomas, the subsequent mutational activation of the *KRAS* oncogene , and the inactivation of the genes encoding *p53*[10]. Recent studies suggested crosstalk between gene mutations (*i.e*., mutations of *BRAF*, *KRAS*, and *p53* and microsatellite instability) and epigenetic alterations (*i.e.,* DNA methylation of CpG island promoter regions) in cancer development; in fact, gene mutations could potentially affect epigenetic patterns and epigenetic changes could guide mutation processes and genome instability[11]. CRC can be considered a sporadic disease in most cases (75%-80%), as a consequence of the accumulation of both mutations and epigenetic alterations of numerous genes[12]. A number of studies on DNA methylation concluded that there are no less than three subtypes of CRC in relation to the rate of DNA methylation and mutations in key genes for CRC[13]. The interaction of both gene mutations and epigenetic changes could be responsible for the development of malignant adenocarcinomas as a result of the interference on signaling pathways that control cell growth and tumor progression[14]. Currently, research has moved towards the identification of mutations in key genes involved in the progression of cancer (*i.e., APC*, *CTNNB1*, *BRAF* and *KRAS*), which are implicated in the WNT and the RAS-RAF-mitogen-activated protein kinase (MAPK) signaling cascades, and, eventually, in the classical adenoma-carcinoma sequence pathway (Table 1). The aim of this review was to summarize the most recent evidence on the possible use of genetic or epigenetic biomarkers for diagnosis, prognosis and response to therapy in CRC patients.

TISSUE- BASED BIOMARKERS

BRAF

BRAF is a gene that encodes a serine-threonine protein kinase and is a regulator of the MAPK pathway that is located downstream of *KRAS*. *BRAF* represents a prognostic biomarker and a possible target for therapies in patients with CRC[15]. Activating mutations of *BRAF* occur most frequently in codon 600, and are demonstrable in different types of cancers, for example CRC (10%), melanoma (50%)[16], and lung tumors (1%–2%)[17]. The conversion of valine 600 to glutamic acid (V600E) accounts for 80% of the *BRAF* mutations in CRC. There is evidence that *KRAS* and *BRAF* mutations are mutually exclusive events in cancer progression and

Table 1 Examples of biomarkers for colorectal cancer diagnosis, progression, prognosis and treatment

Biomarker		Prognostic factor	Predictive factor
BRAF mutations	Specific phenotype and metastasis; resistance to anti-EGFR mAb	Yes[6,110]	Yes[111], Potentially[6,110]
KRAS mutations	Heterogeneity of CRC; resistance to anti-EGFR mAb	Yes potentially[110]	Yes[6,110,111]
MSI	Resistance to 5-FU	Yes[6,110], No[111]	-
APC mutations	Poorer overall survival	Yes[66]	Yes[65]
Micro-RNA	Early detection of CRC, prognostic stratification and therapy-response prediction	Yes[72]	Yes[73]
PIK3CA mutations	Poor prognosis and particular clinico-pathological characteristics; resistance to anti-EGFR mAb	Yes[82]	Yes[110]
Loss of *PTEN*	High tendency to develop metastasis; Resistance to anti-EGFR mAb	-	Yes potentially[110,111]
TP53 expression	Poor prognosis	Yes potentially[110], No[111]	-
Loss of *NDST4*	Adverse prognosis; molecular predictor of metastasis	Yes[95]	Yes[95]
Loss of *18qLOH*	Poor prognosis	Yes[111], Potentially[110]	-
IGFR-1R	High levels in metastastic CRC; poor overall survival	Yes[104]	Yes[104]

National Comprehensive Cancer Network Guidelines[6]; European Society for Medical Oncology Guidelines[110]; American Society of Clinical Oncology Guidelines[111]. CRC: Colorectal cancer; EGFR: Epidermal growth factor receptor; MSI: Microsatellite instability; FU: Fluorouracil.

development[18]. Many studies highlighted different responses to anti-EGFR treatment according to *BRAF* status, with a failing rate of anti-EGFR up to 12%-15% in *BRAF* (V600E) mutation carriers[19]. Some studies showed a high methylation rate (CIMP-high) in *BRAF* mutation carriers compared to *BRAF* wild-type (WT) cancer; furthermore, it has been demonstrated a marked association between BRAF mutation and MSI[20]. BRAF mutant cancers are characterized by high prevalence in women and in elderly patients (> 70 years)[21], four or more positive lymph nodes, high-grade histology, defective mismatch repair status, and are mainly sited in the right side of the colon, while wild type tumors can generally affect any part of the colon and rectum[22]. Many retrospective studies underlined the poor prognosis in patients with *BRAF* mutations. Roth *et al*[23] evaluated the prognostic role of *KRAS* and *BRAF* in 3278 patients with stage Ⅱ and Ⅲ colon cancer patients receiving irinotecan added to fluorouracil (FU)/leucovorin (FA) as adjuvant treatment. The results confirmed that the *KRAS* mutation status does not have significant prognostic value, while *BRAF* is prognostic for overall survival in MSI low and stable tumors, especially in stage Ⅱ patients[23]. Similar results were observed in a study by Yokota *et al*[24] carried out in 229 patients on the prognostic impact of *KRAS*/*BRAF* mutations in advanced and recurrent CRC patients receiving chemotherapy treatment. *KRAS* and *BRAF* mutations were observed in 34.5% and 6.5% of patients, respectively. The overall survival in patients with *KRAS* and *BRAF* mutations (27.7 and 11.0 months respectively) was significantly poorer than that observed in patients with wild type forms of these genes. The results confirmed that *BRAF* mutations can be considered a strong prognostic factor for poor survival in advanced and recurrent CRC[24]. Nowadays, there is growing interest in the understanding of treatment implications of *BRAF* mutations. The MRC FOCUS trial evaluated the effects of FU, FU/irinotecan or FU/oxaliplatin administration in in 711 patients with advanced CRC and showed, as previously reported, that patients with *BRAF* mutations had a lower overall survival compared to patients with *BRAF*-WT. It is noteworthy that the response to chemotherapy treatment was not influenced by *BRAF* status, suggesting that *BRAF* mutations should not be considered as predictive biomarkers for irinotecan or oxaliplatin[25]. A number of studies highlighted that *BRAF* mutations in CRC can predict the lack of response to anti-EGFR treatment. Bokemeyer *et al*[26] analyzed pooled individual patient data from the CRYSTAL and OPUS randomized clinical trials (RCTs). The results of these RCTs showed that when cetuximab was added to first line chemotherapy treatment in patients with *KRAS*-WT CRC, there was a significant improvement in overall survival, progression-free survival, and best overall response rate. No significant differences were observed in the outcome between *BRAF* mutation carriers and *BRAF*-WT receiving EGFR-targeting therapies. Patients with *BRAF* mutations had a poorer prognosis compared to those with BRAF-WT[26]. A meta-analysis by Mao *et al*[27] carried out on 11 studies (7 studies for unselected mCRC patients and 4 studies for patients with *KRAS*-WT metastatic CRC),demonstrated that the *BRAF*(V600E) mutation is related to a lack of response in *KRAS*-WT metastatic CRC patients receiving anti-EGFR monoclonal antibodies. Another meta-analysis that included 463 patients with *RAS*-WT/*BRAF* mutant status CRC reported similar results. The analysis included 9 phase Ⅲ trials and 1 phase Ⅱ trial (6 first-line and 2 second-line trials, plus 2 trials involving chemorefractory patients). The addition of anti-EGFR monoclonal antibodies cetuximab and panitumumab in the *BRAF* mutant subgroup did not lead to any improvement in outcome compared to standard therapy or best supportive care. These results underlined the importance of *BRAF* mutation evaluation before starting anti-EGFR monoclonal antibody therapies[28]. Because of their growing importance, the NCCN guidelines strongly recommend *BRAF* and *RAS* (*KRAS* exon 2 and non-exon 2; *NRAS*) mutation testing for diagnosis of stage Ⅳ CRC[6]. Based on this evidence, *BRAF* mutations may be used as a biomarker to screen metastatic CRC

patients who could benefit from therapy with anti-EGFR antibodies.

KRAS

The *KRAS* gene encodes a small GTPase transductor protein that regulates cellular growth and differentiation[29]. The *KRAS*-WT protein is transitorily activated during signal transduction, but mutations in the *KRAS* gene could lead to the continuous activation of this signal transduction pathway and, as a result, to cell transformation and inefficacy of therapy with anti-EGFR antibodies[14]. Most activating mutations of *KRAS* involve codons 12 (82%-87%) and 13 (13%-18%), and only rarely codons 61, 63 and 146. Mutations in codon 12 are linked to mucinous CRC, while mutations in codon 13 are predominantly non-mucinous, showing more aggressive behavior and a tendency to develop metastasis[30]. A number of studies pointed out the key role of *KRAS* mutations as predictive markers for anti-EGFR therapy. An open-label, randomized, multicenter, phase Ⅲ study by Van Cutsem *et al*[31] showed that the administration of cetuximab in patients with metastatic *KRAS*-WT CRC receiving irinotecan, FU, and leucovorin (FOLFIRI) resulted in significant benefits as regards overall survival, progression-free survival and response compared with FOLFIRI alone. These results confirmed the importance of *KRAS* mutation status as a strong predictive biomarker for the efficacy of cetuximab plus FOLFIRI[31]. The benefits from cetuximab in advanced CRC patients with *KRAS*-WT, but not in those with *KRAS* mutation, were also reported in a RCT by Karapetis *et al*[32]. The Authors analyzed tumor samples from 394 patients with CRC, who were given cetuximab plus best supportive care (BSC) or BSC alone. Of these patients 42.3% showed at least one mutation in exon 2 of the *KRAS* gene. In CRC patients with *KRAS*-WT, cetuximab significantly improved overall survival and progression-free survival when compared with BSC alone. These differences were not observed in CRC patients with *KRAS* mutations. The presence of *KRAS* mutations represents a negative predictive factor, and plays a crucial role in the decision about the use of anti-EGFR therapy (Figure 1).

Microsatellite instability

Microsatellites are short tandem repeats of DNA sequences positioned throughout the human genome. MSI is a hypermutable phenotype caused by a deficient DNA mismatch repair (MMR) system, mainly due to the inactivation of the four MMR genes (*MSH2*, *MLH1*, *MSH6* and *PMS2*) that leads to a failure in the correction of the insertion or the deletion of repeating units during DNA replication [33]. MSI is observed in about 15% of all CRCs; 3% of these are associated with Lynch syndrome (Hereditary non polyposis colorectal cancer or HNPCC), and the other 12% are due to sporadic, hypermethylation of the promoter of the *MLH1* gene, in patients with the CpG island methylator phenotype[34]. CRCs with microsatellite instability are more frequent in the right colon, are mucinous with signet ring cell morphology, show poor differentiation and strong lymphocyte infiltration. Overall, CRC patients with MSI have a better prognosis that those without MSI and show a different response to chemotherapy treatment[35]. It has been observed that stage Ⅱ or stage Ⅲ CRC patients with stable or low MSI could benefit from adjuvant chemotherapy with 5-fluorouracil, while patients with stage Ⅱ CRC and high MSI show a 3-fold increase in mortality, probably due to the immunosuppressive effects of the therapy[36]. On the contrary, a retrospective study carried out by Fallik *et al*[37] on a small number of patients (n = 72) with metastatic CRC showed that the administration of irinotecan could be useful in MSI tumors even if these results need further clarification and are not yet applicable in routine clinical practice. A meta-analysis of eight independent studies conducted by Des Guetz *et al*[38] included a total of 287 patients who received 5FU-based chemotherapy, and 678 patients who were treated with other chemotherapy regimens (5FU or capecitabine with oxaliplatin and/or irinotecan). The data were analysed with a random-effect model due to heterogeneity between studies. The authors concluded that MSI status is not a predictive factor for the effect of chemotherapy, with comparable results in both MSI-High and MSI-stable metastatic CRC tumors[38]. MSI can be considered a promising prognostic marker for CRC patients and MSI status can be assessed through a panel of five markers (*BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S2720*) and the use of polymerase chain reaction (PCR). MSI-high is characterized by instability at two loci or more, and MSI-Low by instability at one locus[39]. Currently, the main clinical use of MSI testing is to detect patients with Lynch syndrome. About 15% of all CRCs show MSI, and among these 75%–80% are characterized by acquired methylation of *MLH1*; 2%–3% of all CRCs show germ-line mutations in one of the MMR genes[40]. There is growing interest in MSI testing as regards the adjuvant setting to guide therapeutic choices; however, the implication of MSI in the metastatic setting is not well recognized.

EPIGENETIC MARKERS IN CRC

CpG island methylator phenotype

The term "epigenetics" refers to modifications in the phenotype or gene expression that do not implicate DNA sequence changes. Among these, DNA methylation is one of the most studied CRC biomarkers and plays a pivotal role in the alteration of gene expression observed in cancerogenesis[41] (Table 2). Hypermethylation of CpG islands sited in the promoter regions of tumor suppressor genes is a well-recognized mechanism for gene inactivation[42]. The inactivation of gene transcription is due to changes in the chromatin structure of a gene promoter that becomes inaccessi-

Table 2 Examples of epigenetic biomarkers for colorectal cancer

Epigenetic markers	
Methylated genes/loci	
p14 ARF, p15 INK 4b, p16 INK4a	Cell cycle regulation
hMLH1, MGMT	DNA repair system; progression from adenoma to cancer
DAPK	Apoptosis
THBS1	Angiogenesis inhibition
SPARC	Lymphovascular invasion, metastasis
TIMP3	Metastasis suppression
CDH1, CDH13	Cell adherence
Methylation biomarkers	
VIM, SEPT9, SFRP2	Biomarkers for CRC and as DNA-based colon cancer screening tests
TWIST1, IGFBP3, GAS7, ALX4, SDC2	Higher methylation levels in CRC compared to normal subjects (promising diagnostic biomarkers)
Candidate biomarkers	
Methylated *UGT1A1*	Affects irinotecan treatment (*in vitro*)
Methylated *DYPD, UMPK*, and *SPARC*	Affect 5-FU treatment (*in vitro*)
TFAP2E	No responsiveness to oxaliplatin, irinotecan, and 5-FU

CRC: Colorectal cancer; FU: Fluorouracil.

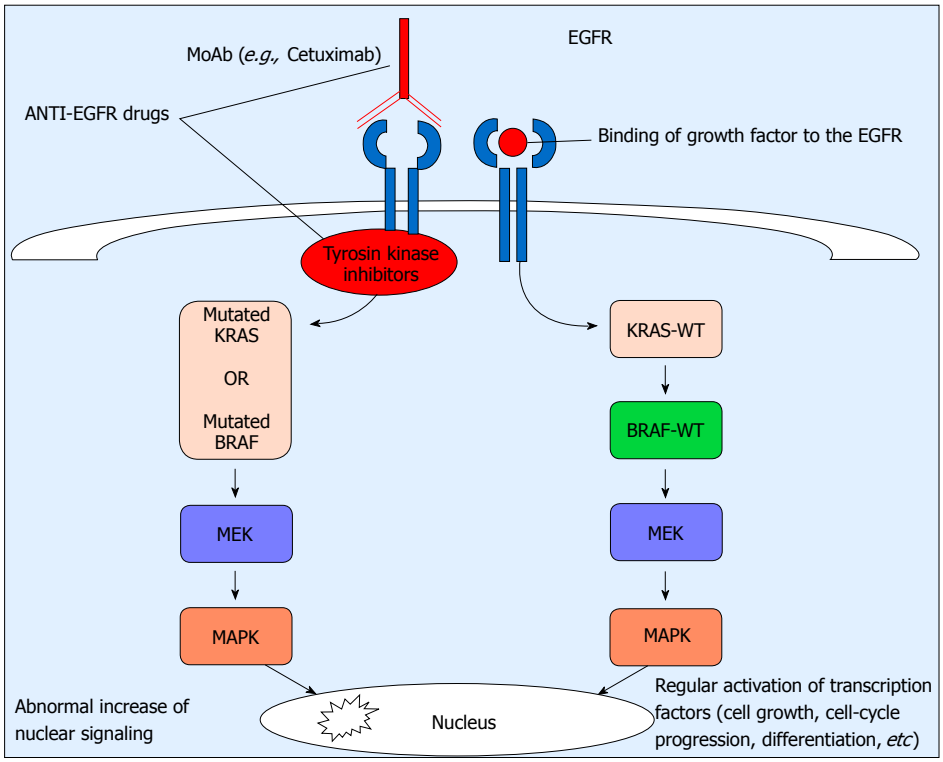

Figure 1 Epidermal growth factor receptor pathway in patients with wild-type and mutant *BRAF* and *KRAS*. On the right side of the figure, the normal epidermal growth factor receptor (EGFR) pathway is characterized by the binding of growth factor to the EGFR that leads to regular activation of transcription factors and cell-cycle progression; On the left side, mutations in *BRAF* or *KRAS*, which are mutually exclusive, cause the activation of the EGFR pathway and therefore an abnormal increase of nuclear signaling and no response to monoclonal antibodies. EGFR: Epidermal growth factor receptor; MEK: Mitogen-activated protein kinase kinase; MAPK: Mitogen-activated protein kinase; MoAb: monoclonal antibodies; WT: Wild type.

ble to transcription factors[42]. This epigenetic alteration is able to inactivate a number of cellular pathways that include, for example, DNA repair system (*hMLH1*, *MGMT*), apoptosis (*DAPK*), angiogenesis inhibition (*THBS1*), metastasis suppression (*TIMP3*), cell cycle regulation (*p14 ARF*, *p15 INK 4b*, *p16 INK4a*), and cell adherence (*CDH1*, *CDH13*)[43,44]. There is evidence of a strong correlation between CIMP-high and right colon cancer, microsatellite instability and a high rate of *BRAF* mutation[44]. Some studies showed that abnormal methylations of DNA repair genes, for example, *MGMT* and *MLH1*, may lead to the progression from adenoma to cancer. The mechanisms involved are the creation of a more prone state for G->A mutations, as frequently observed in *KRAS* in the case of methylated *MGMT* and a favorable condition for MSI in the case of methylated *MLH1*[45,46]. Lee *et al*[47] suggested that the CIMP could be used as a predictive marker for anti-EGFR therapy.

However further prospective studies are needed to confirm this hypothesis. Methylated genes such as *MLH1*, *VIM* and *SEPT9*, could be used as biomarkers for colorectal cancer and as DNA-based colon cancer screening tests. Methylated Vimentin (mVim) is a validated stool-based biomarker for early detection of colorectal cancer available in the US (ColoGuard assay; LabCorp)[48]. The methylated *VIM* gene is observed in most CRC (53%–84%). The test is PCR based and is able to measure methylated *VIM* and DNA integrity with high sensitivity and specificity (83% and 82%, respectively)[49]. A recent meta-analysis of 25 studies by Nian *et al*[50] pointed out that methylated *SEPT9* (Epi proColon; Epigenomics AG) can be considered as an effective blood-based assay in CRC detection, mostly for advanced tumors. The proportion of heterogeneity due to threshold effect was 0.02, which indicated the absence of significant thread hold effect among the included studies. Meta-regression demonstrated that study types, country (Asian population or not), sample size (less or greater than 300) kits used (Epipro colon or not), and risk of bias of included studies were all sources of heterogeneity of sensitivity and specificity[50]. Perez-Carbonell *et al*[51] carried out a systematic analysis of a panel of methylated CRC-specific genes (*SEPT9*, *TWIST1*, *IGFBP3*, *GAS7*, *ALX4* and *miR137*) and observed that methylation levels of all these genes were significantly higher in CRC compared to normal subjects ($P < 0.0001$), mainly as regards miR137 and *IGFBP3* (86.7% and 83%, respectively). The combination of these two genes showed a sensitivity of 95.5% and a specificity of 90.5% for the detection of CRC, thus representing a promising diagnostic biomarker. Moreover, the results of this study underlined that hypomethylation of *IGFBP3* could represent an independent risk factor for poor prognosis in patients with stage Ⅱ and Ⅲ CRC. Interestingly, in stage Ⅱ and Ⅲ CRC patients who showed hypermethylation of *IGFBP3*, adjuvant chemotherapy with 5FU did not improve overall survival or disease free progression[51]. Methylated *IGFBP-3* could be used as a potential target for the development of novel anticancer drugs, for example demethylating agents. Further studies are needed to clarify the association between methylated *IGFBP-3* and low recurrence-free survival, and to report the efficacy of demethylating agent alone or combined with adjuvant therapy in CRC patients[52]. A study by Tang *et al*[53] underlined the importance of methylated secreted frizzled-related protein 2 (*SFRP2*) as a possible marker for CRC detection and staging. *SFRP2* can be isolated from CRC tissues, serum and fecal DNA, with sensitivity for CRC that ranges from 66.9% to 88.2%. A higher specificity of *SFRP2* methylation levels for CRC was observed in serum compared to tissue and stool DNA. Furthermore, there was a significant association of serum *SFRP2* with low differentiation grade, serosal or subserosal infiltration, lymph node metastasis and TNM stage of CRC[53]. Other promising blood biomarkers include methylated thrombomodulin (*THBD*) that detected 71% of all CRCs at a specificity of 80%[54], and methylated syndecan 2 (*SDC2*) that showed a sensitivity of 92% for CRC at stage Ⅰ[55]. There is emerging evidence that epigenetic mechanisms could affect the response to chemotherapy[56]. Increased thymidylate synthetase (*TYMS*) expression, which is regulated by histone acetylation and deacetylation, seems to be the main mechanism involved in the development of resistance to 5-FU. A study by Watson *et al*[57] showed that CRC patients with *TYMS* amplification receiving post-resection 5-FU-based chemotherapy, showed shorter median survival. Other genes involved in pyrimidine metabolism that could determine resistance to 5-FU, thus guiding the chemotherapy choice for CRC, include thymidine phosphorylase (*TYMP*), uridine monophosphate/cytidine monophosphate kinase (*UMPK*), and dehydrogenase (*DYPD*) genes[53]. A clinical trial by Ebert *et al*[58] examined an initial cohort of 74 patients, followed by four cohorts of patients (total $n = 220$) and showed that CRC patients with high levels of methylation of the gene encoding transcription factor AP-2 epsilon (*TFAP2E*) did not benefit from chemotherapy treatment with 5-FU, irinotecan or oxaliplatin ($P < 0.001$). *TFAP2E* resistance is mediated through its downstream target gene DKK4, encoding dickkopf homolog 4 protein. In CRC patients with *TFAP2E* hypermethylation, targeting of *DKK4* could represent a possible option to bypass the resistance to chemotherapy mediated by *TFAP2E*[58]. Some studies showed a possible association between methylation of the *SPARC* gene (coding for the matricellular protein osteonectin)[59], and methylation of the *UGT1A1* gene (coding for the UDP glucuronosyltransferase-1A1 enzyme)[56] to a reduction of chemosensitivity to 5-FU or irinotecan. Amatu *et al*[60] carried out a phase Ⅱ study with dacarbazine in CRC patients who did not respond to standard chemotherapy (oxaliplatin, irinotecan, fluoropyrimidines, and cetuximab or panitumumab if *KRAS*-WT). Dacarbazine is an antineoplastic alkylating agent that acts by DNA methylation and causes base pair mismatch. Considering all CRC patients, 40% present hypermethylation of the *MGMT* gene and dacarbazine is effective only in tumors that lack *MGMT*[60].

APC

Adenomatous polyposis coli (*APC*) is a suppressor gene that was detected by genetic linkage analysis in familial adenomatous polyposis (FAP). Mutated APC is also responsible for most sporadic CRCs[61]. *APC* acts as an antagonist of the WNT signaling pathway and regulates many cell activities such as migration and adhesion, transcriptional activation, and apoptosis[62]. Around 70%-80% of patients with CRC show the loss of *APC*[63]. A meta-analysis by Liang *et al*[64] evaluated the associations between three APC polymorphisms (*D1822V*, *E1317Q*, and *I1307K*) and the risk of CRC.

The results showed a low association between *E1317Q* and the risk of CRC, especially for adenomas. Ashkenazi Jews *I1307K*-variant carriers showed a significantly increased risk of CRC compared to *I1307K* wild-type carriers. In this meta-analysis, there was no evidence of heterogeneity between studies; however, all the included studies were case-control studies with high likelihood of recall bias and selection bias[64]. Another recent meta-analysis highlighted that hypermethylated *APC* promoter was more frequent in adenoma than in normal control samples. Moreover, *APC* hypermethylation levels were higher in CRC patients at stage Ⅰ compared to normal controls. The authors concluded that *APC* hypermethylation could represent an important biomarker for early CRC diagnosis and a possible treatment target for personalized therapy. Interestingly, the results did not show a significant association between APC promoter methylation and overall survival in CRC patients. The heterogeneity in the meta-analysis was 43%, and there was no publication bias. However, only publications in English and Chinese were included in the study, thus suggesting the possible existence of selection bias[65]. Another study showed that *APC* mutation/high miR-21 in patients with advanced CRC had poorer overall survival. The mutation of *APC* and expression of miR-21 might be useful clinical predictors for CRC[66-68].

microRNA

microRNAs (miRNAs) are small non-coding RNA sequences that can control the expression of genes at the post-transcriptional level[69]. miRNAs play crucial roles in cancer biology and are involved in a number of cellular processes such as proliferation, apoptosis, differentiation, invasion and metastasis[70]. There is growing evidence that carcinogenesis and tumor progression could be associated with abnormalities of miRNAs[71]; thus, miRNAs could represent valuable biomarkers for early detection of cancer, prognostic stratification and therapy-response prediction[72,73]. miRNAs can be isolated from a variety of biological samples, including blood, saliva and stools[74]. A recent study identified a set of 19 differentially expressed miRNAs. Among these, the up-regulation of hsa-miR-183-5p and hsa-miR-21-5p, and the down-regulation of hsa-miR-195-5p and hsa-miR-497-5p were associated with CRC through the interplay with the *MMR* pathway and transforming growth factor β, *WNT*, *RAS*, *MAPK*, and *PI3K* signaling pathways[68]. miR-21 is one of the most studied miRNAs[75]; a recent meta-analysis by Peng *et al*[67] investigated the role of miR-21 in CRC and reported a sensitivity of 0.64, a specificity of 0.85 and an area under the curve of 0.85, as regards diagnostic test accuracy. Samples taken from blood circulation showed corresponding values of 0.72, 0.84, and 0.86 respectively. As regards diagnostic meta-analysis of miR-21-related combination biomarkers, the above values were 0.79, 0.79 and 0.86, respectively. The highest predictive power was observed for miRNA combination markers in circulation (0.85, 0.86, and 0.92 respectively). These results suggested that circulating (especially in serum) miR-21 could represent a promising diagnostic biomarker, while tissue miR-21 could be a useful prognostic marker for CRC. Meta-regression analysis found that ethnicity, sample size, and sample source did not have a significant effect on the pooled results ($P > 0.10$). Also, there was no heterogeneity from the threshold effect.

OTHER PROMISING BIOMARKERS FOR CRC

Phosphatidylinositide-3-kinases

Phosphatidylinositide-3-kinases (PI3K) are lipid kinases that are involved in the regulation of cellular behavior, including proliferation, adhesion and survival[76]. PI3K signaling is a major pathway for *RAS*-mediated proliferation, transformation and tumor progression[77]. Abnormalities in PI3K signaling are frequently observed in human cancer[78] and mutations in the *PIK3CA* gene, the gene coding for the catalytic subunit p110alpha of PI3K, have been described in many cancers, including CRC[79]. These mutations in CRC are associated with right side location, mucinous histological type, *KRAS* mutation, loss of *MGMT* expression and a high degree of methylation (CIMP)[78]. *PIK3CA* mutation is also associated with a significant reduction in survival in CRC patients with *BRAF*-WT[80]. Mutations at *PIK3CA* exon 9 and exon 20 trigger different biologic effects and are responsible for the promotion of cancerogenesis. A genetic and biochemical analysis conducted by Zhao *et al*[81] demonstrated that coexistent mutations in both exons 9 and 20, but not in exon 9 or 20 alone, result in an increase of tumorigenic effects with worse cancer-specific survival. Jehan *et al*[82] analyzed data from 220 patients who received adjuvant chemotherapy and/or radiotherapy and showed that *PI3KCA* amplification could represent an independent prognostic marker for better survival and a promising marker to detect CRC patients that may benefit the most from adjuvant therapy. Recent studies proposed mutated *PIK3CA* as a biomarker to detect CRCs sensitive to aspirin. Liao *et al*[83] carried out a study in 964 patients with CRC and observed that patients with mutated-*PIK3CA* who started aspirin therapy after diagnosis, showed higher colorectal cancer-specific survival (multivariate hazard ratio for cancer-related death, 0.18; 95%CI, 0.06 to 0.61; $P < 0.001$ by the log-rank test) and overall survival (multivariate hazard ratio for death from any cause, 0.54; 95%CI, 0.31 to 0.94; $P = 0.01$ by the log-rank test), as compared to patients with *PIK3CA*-WT[83]. Domingo *et al*[84] studied 896 participants in the Vioxx in Colorectal Cancer Therapy: Definition of Optimal Regime trial, and confirmed the role of mutated *PIK3CA* as a predictive molecular biomarker in CRC patients for adjuvant aspirin therapy. A population-

based cohort study of 740 stage Ⅱ and Ⅲ CRC patients, showed a 31% improvement in cancer-specific survival in aspirin users compared to non-users (adjusted HR = 0.69, 95%CI: 0.47–0.98). These outcomes were more evident in patients with high *PTGS2* (prostaglandin-endoperoxide synthase 2, also known as cyclooxygenase-2 or COX-2) expression compared to those with low *PTGS2* expression. Further trials are needed to better detect CRC patients who may receive a survival benefit from aspirin therapy[85].

PTEN

PTEN is a tumor suppressor gene that regulates the cell-survival signaling pathway initiated by PI3K. *PTEN* mutations are associated with advanced and metastatic tumors[86] and *PTEN* promoter hypermethylation is frequently observed in MSI-high sporadic CRCs[87]. Patients with *PTEN* expression showed significantly longer overall survival compared to patients with *PTEN* loss tumor[88]; other studies reported an association with poor prognosis in stage Ⅱ patients only[86] or in CRC patients with liver metastasis[89]. *PTEN* could represent a useful predictive marker for *KRAS*-WT patients treated with anti-EGFR therapy[90].

TP53

The *TP53* gene encodes a tumor suppressor protein that is involved in the regulation of cell cycle, apoptosis, senescence, and DNA repair. *TP53* mutations may result in altered function of TP53 protein, which plays a pivotal role in tumorigenesis. *TP53* mutations are observed in about 60% of colorectal tumors and can be found in both adenomas and in malignant cells[91]. There is evidence that the expression of *p53* mRNA could represent a useful predictor of survival in patients with stage Ⅲ CRC or rectal cancer[92].

NDST4

NDST4 is a tumor suppressor gene located at chromosome 4q26. Most CRCs showed a significant decrease in *NDST4* expression compared to normal colonic mucosa and some studies showed that the loss of *NDST4* was associated with higher pathological stages and poor survival[93]. *NDST4* belongs to the N-deacetylase/N-sulfotransferase (heparan glucosaminyl) (*NDST*) family, and regulates heparan sulfate (HS) biosynthesis on a core protein to form heparan sulphate proteoglycans (*HSPGs*)[94]. The loss of *NDST4* function could lead to an increase in the invasive ability of cancer cells through changes of the interaction between cell adhesion receptors and their ligands. The genetic loss of *NDST4* could represent a biomarker of adverse prognosis for patients with CRC[95].

Chromosome 18q loss of heterozygosity

Loss of heterozygosity of chromosome 18q (18qLOH) is a genetic alteration frequently observed in CRC[94] and many key genes (*i.e., DCC*, *SMAD2* and *SMAD4*), involved in CRC tumorigenesis, are located on chromosome 18q[95,96]. A study by Sarli *et al*[97] carried out in 118 patients, reported a decreased overall survival for patients with CRC stage Ⅲ and 18qLOH compared to non-18qLOH patients. The authors concluded that 18qLOH could represent an informative genetic marker, and has the potential to be used to predict recurrences and survival in resected stage Ⅲ CRCs[97]. A meta-analysis of 27 studies on the prognostic significance of 18q LOH showed that chromosome 18q allelic imbalance and *DCC* loss of expression could be considered as negative predictive factors for survival. In this metanalysis there was evidence of significant heterogeneity and publication bias[98]. However, these findings suggested that 18q LOH/*DCC* status could help to detect CRC patients who may benefit from adjuvant chemotherapy after potential curative surgery[99]. Boulay *et al*[100] analyzed 202 colorectal tumour biopsies from a previous randomised study of adjuvant chemotherapy, and observed that patients with the loss of 18q (and *SMAD4* deletion) could obtain less benefit from adjuvant 5-FU treatment.

IGFR-1R

The type 1 insulin-like growth factor receptor (IGF-1R) is a transmembrane glycoprotein composed of two extracellular subunits and two cytoplasmic subunits with tyrosine kinase activity. Overexpression of IGFR-1R has been observed in various tumors (*i.e.,* primary renal cancer cells, and preinvasive breast lesions), and its activation is involved in cell proliferation, differentiation, angiogenesis, and apoptosis[101,102]. IGF-1R undergoes nuclear translocation and interacts with chromatin, under the regulatory effect of IGF[103]. IGF-1R has become a target of new treatments, especially monoclonal antibodies or tyrosine kinase inhibitors. In vitro studies demonstrated that chemotherapy resistance in CRC cell lines was associated with overexpression of IGF-1R within the nuclear compartment. Recently, Codony-Servat *et al*[104] carried out a study in four cohorts of patients with metastatic CRC (total *n* = 470), and showed that IGF-1R nuclear location might lead to chemotherapy and targeted agent resistance. Metastatic CRCs presented higher levels of IGF-1R compared to untreated primary cancers and showed poor overall survival. It is noteworthy that ganitumab, an IGF-1R blocking monoclonal antibody, and dasatinib, an SRC inhibitor, augmented the nuclear localization of IGF-1R. Based on these results, IGF-R1 could represent a new potential biomarker for poor prognosis in patients with metastatic CRC[104].

CONSENSUS MOLECULAR SUBTYPES CLASSIFICATION OF CRC

The consensus molecular subtypes (CMS) classification is a recent CRC classifications based on comprehensive gene expression profiling[105,106]. CRC can be separated

into 4 groups called *CMS1*, *CMS2*, *CMS3* and *CMS4*, and each group shows a unique biology and gene expression pattern: *CMS1* (MSI immune, 14%), with higher mutation levels, presence of MSI and marked immune activation; *CMS2* (canonical, 37%), found in epithelial CRCs, with higher *CIN*, and strong *WNT* and *MYC* signaling activation; *CMS3* (metabolic, 13%), observed in epithelial CRCs with evident metabolic disorders; and *CMS4* (mesenchymal, 23%), with noticeable TGF-beta activation, angiogenesis and stromal invasion. The remaining 13% may show mixed characteristics due to transition phenotype or intratumoral heterogeneity[106]. A recent retrospective study by Okita *et al*[107] carried out in 193 patients with metastatic CRCs, showed that the biological features of CMS may affect the efficacy of chemotherapy. In fact, the results of the study demonstrated that in *CMS4* subtype, chemotherapic regimens containing irinotecan showed more benefit than those containing oxaliplatin for progression-free survival [hazard ratio (HR) = 0.31, 95%CI: 0.13-0.64] and overall survival (HR = 0.45, 95%CI: 0.19-0.99). As regards anti-EGFR therapy, *CMS1* showed worse progression-free survival (HR = 2.50, 95%CI: 1.31-4.39) and overall survival (HR = 4.23, 95%CI: 1.83-9.04), while *CMS2* had better progression-free survival (HR = 0.67, 95%CI: 0.44-1.01) and overall survival (HR = 0.49, 95%CI: 0.27-0.87) compared to the other subtypes[107]. There is evidence that CMS classification could represent the starting point for future clinical stratification and subtype–based targeted interventions for CRC. A study by Isella *et al*[108] identified 5 CRC intrinsic subtypes (CRIS) characterized by unique molecular, functional and phenotypic features: (1) CRIS-A: mucinous subtype, glycolytic metabolism, with marked MSI, mutated BRAF or KRAS; (2) CRIS-B: active TGF-β signaling, epithelial–mesenchymal transition, bad prognosis; (3) CRIS-C: high EGFR signaling, and sensitivity to EGFR inhibitors (*i.e.,* cetuximab); (4) CRIS-D: high WNT signaling, *IGF2* gene amplification/overexpression (which has been involved in reduction of sensitivity to EGFR blockade in patients with *KRAS*-WT CRCs)[109]; and (5) CRIS-E: Paneth-like phenotype and *TP53*-mutated genotype. CRIS subtypes categorized independent groups of primitive and metastatic CRCs effectively, representing a great opportunity to enhance patients' management with regard to precision medicine.

CONCLUSION

Research is moving towards a better comprehension of the mechanisms underlying the pathophysiology and the management of colorectal cancer. Recently, new treatment regimens have been developed, mainly for advanced CRC stages. Some of the most useful innovations in the management of CRC include the possibility to detect the absence of *KRAS*, *BRAF*, *NRAS* and *PIK3CA* gene mutations with the subsequent choice to administer targeted adjuvant therapy with anti-EGFR antibodies. Moreover, CRC patients can benefit from tests for MSI and for the detection of 18qLOH that can be helpful in guiding therapeutic decisions as regards the administration of 5-FU. Future therapies for CRC could include targeted therapy against membrane receptors, for example other EGFR ligands, platelet-derived growth factor receptors, and insulin-like growth factor 1 receptor. It seems reasonable to think that in the future, molecular screening will help to recognize patients suitable for specific targeted treatments and to fully characterize cancers. The objective of future research will be to detect biomarkers that could provide a cost-effective and non-invasive diagnosis of CRC; other goals are the identification of the best prognostic panel of biomarkers and the characterization of predictive biomarkers to help in the selection of the most appropriate therapy.

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