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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

New trends in molecular and cellular biomarker discovery for colorectal cancer

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Abstract

Colorectal cancer (CRC) is the third leading cause of cancer death worldwide, which is consequence of multistep tumorigenesis of several genetic and epigenetic events. Since CRC is mostly asymptomatic until it progresses to advanced stages, the early detection using effective screening approaches, selection of appropriate therapeutic strategies and efficient follow-up programs are essential to reduce CRC mortalities. Biomarker discovery for CRC based on the personalized genotype and clinical information could facilitate the classification of patients with certain types and stages of cancer to tailor preventive and therapeutic approaches. These cancer-related biomarkers should be highly sensitive and specific in a wide range of specimen(s) (including tumor tissues, patients' fluids or stool). Reliable biomarkers which enable the early detection of CRC, can improve early diagnosis, prognosis, treatment response prediction, and recurrence risk. Advances in our understanding of the natural history of CRC have led to the development of different CRC associated molecular and cellular biomarkers. This review highlights the new trends and approaches in CRC biomarker discovery, which could be potentially used for early diagnosis, development of new therapeutic approaches and follow-up of patients.

Key words: Colorectal cancer; Biomarkers; Cancer diagnosis; Cancer therapy; Predictive marker; Prognostic marker

Core tip: Colorectal cancer (CRC) is one of the most common leading causes of cancer death in the world; therefore, any attempt in early diagnosis, selection of appropriate therapeutic strategies and efficient follow up can play an important role in reducing the disease related mortalities. Our review highlights the novel trends and approaches in CRC biomarker discovery, which are categorized as pathologic genetic or epigenetic changes within the tumor tissue as well as non-invasive biomarkers such as blood or stool based markers. These biomarkers could be used for the management of cancer patients.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and which is considered for 10% of new cancer diagnoses[1,2]. More than 10% of patients diagnosed with CRC, already have reached to the advanced stages of disease and could show metastasis to the other tissues and organs. Furthermore, about 30% of diagnosed patients with early-stage CRC, have the potential to develop metastatic disease[3].

CRC can be gradually developed through an accumulation of different somatic or inherited changes within genome and epigenome. These pathologic changes lead to the transformation of colonic mucosa into invasive cancer[4]. There are three most important molecular pathways leading to CRC development: (1) Somatic or germ line derived genomic instability due to inactivation of several tumor suppressor genes such as *APC, SMAD4* and *TP53*; aberrant DNA methylation, DNA repair defects induced by mutations in mismatch repair genes (MMR); (2) Mutational inactivation of tumor suppressor genes (*e.g.*, *APC*, *TP53*, *TGFb*, and MMR genes); and (3) Over activation of oncogenic pathways including *BRAF*, *RAS* (*KRAS* and *NRAS*), Phosphatidyl inositol 3-kinase (PIK-3)[5]. The growth and proliferation of metastatic CRC (mCRC) mainly depends on two signaling pathways: the vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR) pathways[6].

The risk of CRC increases with the higher age, as well as by carrying certain inherited genetic mutations (familial adenomatous polyposis and hereditary non-polyposis CRC)[7], a personal or family history of colorectal neoplasia, or having inflammatory bowel disease (IBD)[8].

Cancer related molecular and cellular markers can be classified in four groups: (1) Diagnostic markers, used for risk stratification and early detection; (2) Prognostic markers, give an indication of the likely progression of the disease; (3) Predictive markers, predict treatment response; and (4) Surveillance markers, used to monitor disease recurrence[9].

The increasing disease associated morbidity and mortality, is in part due to a lack of efficient early detection; therefore, earlier diagnosis and more efficient treatment could play a key role in reducing CRC mortality. The conventional examination techniques such as colonoscopy, are invasive; therefore, can affect the patients' willingness for participation in screening programs. Our increasing knowledge about molecular and cellular mechanisms of CRC development, could provide better objectives for management of the cancer patients using different potential biomarkers. Several marker classes have been evaluated for their use in CRC screening and have all shown potential in early phase biomarker studies (Figure 1). Importantly, non-invasive biomarkers derived from biological fluids (blood- or stool-based markers), due to their easy accessibility, could be considered as practical tools for CRC detection and monitoring (Figure 2A). Reliable biomarkers for early detection of CRC, could significantly improve patient prognosis, prediction of treatment response, and possible prediction of recurrence risk (Table 1). Figure 2B, illustrates the approved pipelines for CRC biomarker discovery and validation.

MICROSATELLITE INSTABILITY

Microsatellites are repeating units of DNA sequences (usually 1-6bp in length) that can be found in both non-coding or protein coding sequences of DNA. Microsatellite instability (MSI) is defined as somatic alterations in microsatellite sequences due to the insertion or deletion of those repeat units leading to genomic instability and subsequently increasing the susceptibility for the malformations. Tumors with 10%-29% of unstable microsatellite loci are considered MSI-low (MSI-L) while tumors showing $\geqslant$ 30% of unstable microsatellite loci are known as MSI-high (MSI-H).

MSI generally results from inactivation of the MMR genes through aberrant promoter hypermethylation (80% of MSI CRCs) or mutations in the genes MLH1, MSH2, MSH6, and PMS2 (20% of MSI CRCs)[10]. Inactivation of these genes results in the accumulation of DNA replication errors in microsatellite sequences, importantly those are located in the exons of potential tumor suppressor genes. In sporadic CRC, 10%-15% of tumors display MSI-high. The *MLH1* gene silencing due to the aberrant DNA methylation is responsible for the majority of sporadic CRC with MSI-high[11,12]. A recent meta-analysis assessing the prognostic importance of the MSI in > 7500 patients, could show MSI-H tumors had a better prognosis than MSI-L tumors[13].

Currently, in order to detect MSI in CRC, a specific microsatellite screening panel (Bat-25, Bat-26, MONO-27, NR-21, and NR-24) is used by different clinical laboratories. Therefore, MSI status can serve as a useful predictive and prognostic biomarker for the CRC.

CHROMOSOMAL INSTABILITY

Chromosomal instability (CIN) is defined as the presence of multiple structural or numerical chromosome changes (losses or gains of large portions or whole of the chromosomes) within tumor cells resulting karyotypic

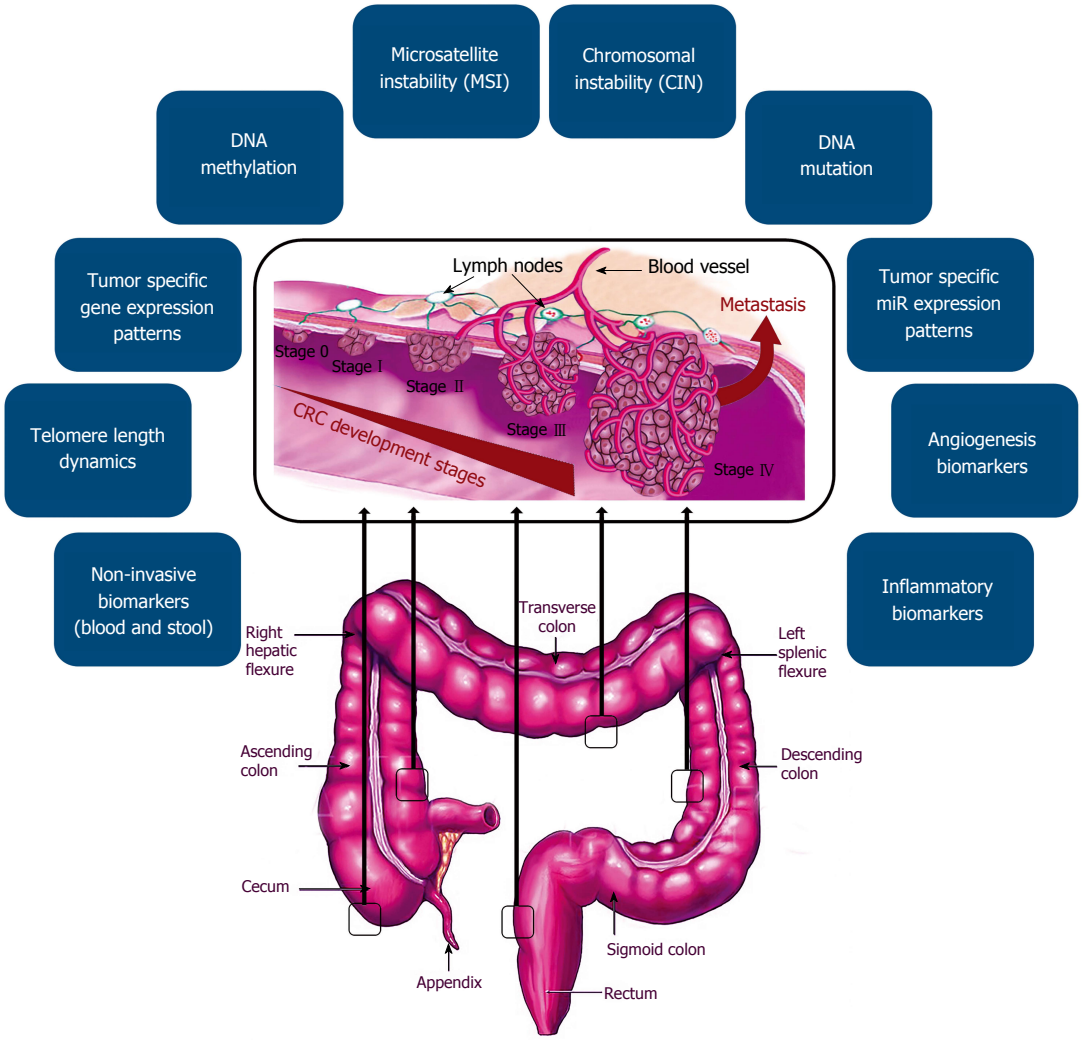

Figure 1 Different classes of colorectal cancer associated molecular and cellular biomarkers.

variability[14]. CIN is the most common form of genetic instability in CRC that present in about 65%-70% of CRCs. The consequence of chromosomal instability is numerical imbalance for chromosomes (aneuploidy), sub-chromosomal amplifications, and also loss of heterozygosity (LOH).

DNA MUTATIONS AS BIOMARKER

Recent data derived from many large-scale high-throughput DNA sequencing approaches, have identified different candidate genes which could have functional roles in the initiation and development of various human cancers[15,16]. Most of these mutations and genetic alterations occur at a relatively low frequency, while some are present in a high portion of tumors.

Analysis of somatic alterations in CRCs by the Cancer Genome Atlas included whole-genome sequencing that identified 24 significantly mutated genes. Commonly observed alterations enable a broad classification into (1) hypermutated tumors (about 15%), of which three-quarters show high-frequency MSI (MSI-H) and one-quarter have somatic mutations in MMR genes and polymerase-ε (POLE) mutations; and (2) nonhypermutated tumors (about 85%) with multiple somatic copy number alterations and aneuploidy that contain activating mutations in KRAS and PIK3CA and loss of heterozygosity of APC and TP53 tumor-suppressor genes[17].

KRAS GENE MUTATIONS

KRAS, a GTPase protein, is encoded by *KRAS* proto-oncogene, which is an early player in many biological pathways. Different point mutations in codons 12 and 13 of exon 2, or mutations in codon 61 of exon 3, lead to constitutive activation of RAS signaling pathway. Therefore, genetic disruption of the *KRAS* gene is one of the essential steps in development of many cancers including CRC.

KRAS is mutated in 30%-50% cases of CRC[18]. According to studies, the adverse impact of *KRAS* mutations on prognosis seems to be stronger in the distal compared with the proximal colon cancers[19]. More than 30% of CRCs carry mutations in exon 2 of

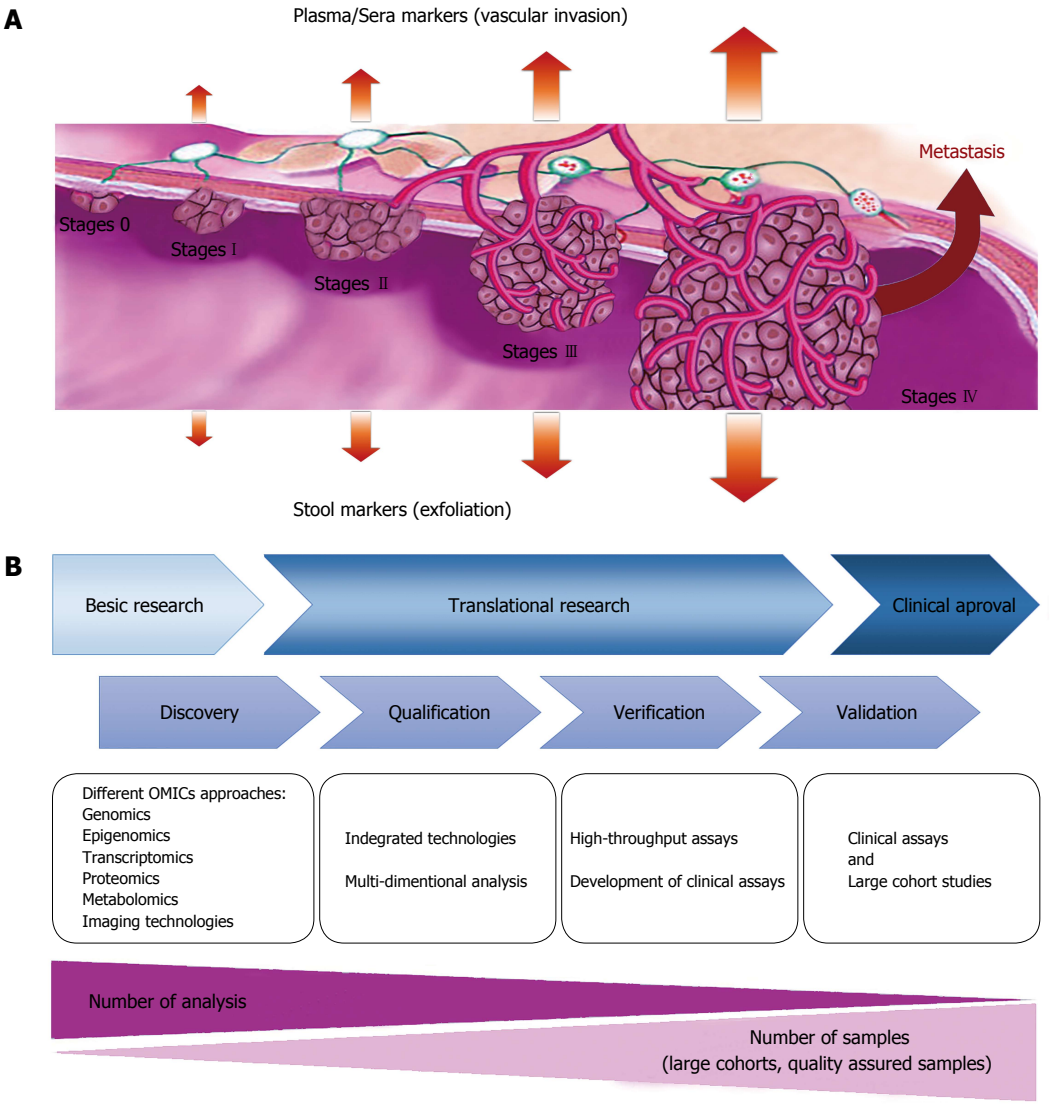

Figure 2 Schematic view of biomarker secretion during different stages of colorectal cancer development (blood and stool biomarkers) (A) and pipelines of biomarker discovery for colorectal cancer (B).

KRAS, and an additional 15% of tumors were found to carry mutations at exons 3 and 4 of KRAS and also exons 2, 3, and exon 4 of NRAS gene[20-22].

Mutations detected in the *KRAS* gene is one of the most utilized predictive marker for response to the anti-EGFR antibody-based therapies using cetuximab and panitumumab[23]. In addition, approximately 60%-70% of metastatic CRC patients with no *KRAS* mutations, show poor response to EGFR antibody therapy, which highlights the contribution of additional mutations in different genes such as *NRAS* in resistance to anti-EGFR treatment[20]. Therefore, the expanded RAS (*KRAS* and *NRAS*) mutation screening is now recommended for all cancer patients before anti-EGFR antibody treatment.

BRAF GENE MUTATIONS

The Raf genes family includes three different serine/threonine kinases (*ARAF*, *BRAF*, and *RAF1*). These protein kinases can activate MEK family proteins (including MEK1 and MEK2), which can further phosphorylate ERK1 and ERK2 proteins. ERK controls cell cycle process *via* regulating enzymes such as Cyclin D1[24]. *BRAF* is the direct downstream effector of *KRAS* within the Ras/Raf/MAPK signaling pathway. *BRAF* gene mutation is reported to be associated with CRC development and also with the poor prognosis of patients[25,26]. Based on the previous studies, *BRAF* gene mutations is associated with aging, female gender, proximal colon location, poor differentiation, mucinous histology, infiltrating lymphocytes and advanced stage of disease[27]. *BRAF* mutations occur more frequently in MSI-H cases of CRC[28].

A subset (about 8%) of CRCs carry a point mutation (V600E) in the BRAF oncogene that is mutually exclusive with mutation in KRAS[29]. Patients whose tumors carry $BRAF^{V600E}$ mutations have been consistently shown to have a poor prognosis in the metastatic setting[30]. In contrast to patients with $BRAF^{V600}$ mutant melanoma[31],

Table 1 Associated biomarkers for colorectal cancer and their predictive value

Biomarkers		Molecular basis	Predictive value	Detection method
Category	**Type**			
Pathological characteristics	Tumor stage Lymph node status Grade of differentiation Anatomy of invasion		Diagnostic, prognostic and predictive markers	Diagnostic radiology and pathological/cytological examination
Proliferation markers	Ki67	Nuclear antigen associated with proliferation	Diagnostic and prognostic markers	IHC
	Cyclins	Regulation of cell cycle phase transition	Diagnostic and prognostic markers	IHC
Chromosome abnormalities	p53	Tumor suppressor gene which shows loss of function	Diagnostic, prognostic and predictive markers	IHC, RT-PCR, FISH
	H-ras, K-ras, N-ras	Membrane-associated GTPase integral to signal transduction cascade, if mutated, causes increased cellular proliferation	Diagnostic, prognostic and predictive markers	IHC, RT-PCR, FISH
	Telomere length	Pathologic telomere length dynamics	Diagnostic and prognostic markers	RT-PCR, Flow cytometry
	Telomerase activity	Maintenance of telomeres and therefore chromosomal length enables progression through successive cell cycles	Diagnostic and prognostic markers	TRAP assay
Hypoxia-regulated genes	HIF-1	HIF-1 transcription factor complex stabilized in hypoxic conditions, leading to transcription of hypoxia-regulated genes	Diagnostic and prognostic markers	IHC
	Glut-1	Increased Glut-1 expression caused by malignant transformation and upregulated by hypoxia. Promotes switch to anaerobic glycolysis to support hypoxic tumor	Diagnostic and prognostic markers	IHC
Angiogenesis	VEGF	Angiogenic growth factor	Prognostic and predictive markers	IHC, FISH, immunoassay
	PD-ECGF	Angiogenic growth factor with thymidine phosphorylase activity	Prognostic and predictive markers	IHC
	Vascularity	New vasculature supports tumor growth	Prognostic and predictive markers	IHC staining for endothelial receptors *e.g.*, CD31, CD34, von Willebrand (factor VIII) combined with measurement of ICD or MVD using digital image analysis techniques
Epigenetics	Aberrant DNA hypermethylation	Inactivation of key tumor suppressor genes including *APC, ATM, BMP3, CDKN2A, SFRP2, GATA4, GSTP1, HLTF, MLH1, MGMT, NDRG4, RASSF2A, SFRP2, TFPI2, VIM,* and *WIF1*	Diagnostic, prognostic and predictive markers	PCR-based methods and Pyrosequencing
	Aberrant DNA hypomethylation	Lids to chromosomal instability and global loss of imprinting	Diagnostic and prognostic markers	PCR based methods and Pyrosequencing
Tumor specific expression patterns	Gene expression patterns	Unique signature of the dysregulated genes/pathways at different forms and stages of CRC	Diagnostic, prognostic and predictive markers	Array-based methods, NGS, RT-PCR
	MicroRNA expression patterns	Unique signature of the dysregulated microRNAs at different forms and stages of CRC	Diagnostic, prognostic and predictive markers	Array-based methods, NGS, RT-PCR

FISH: Fluorescent *in-situ* hybridization; HIF-1: Hypoxia inducible factor-1; HPLC: High pressure liquid chromatography; ICD: Intercapillary distance; IGF-1: Insulin growth factor-1; IHC: Immunohistochemistry; MVD: Microvessel density; NGS: Next generation sequencing; PD-ECGF: Plateletderived endothelial cell growth factor; RT-PCR: Reverse transcription–polymerase chain reaction; RIA: Radioimmunoassay; VEGF: Vascular endothelial growth factor.

CRCs which harbor $BRAF^{V600E}$ mutations were found to be resistant to inhibition of the BRAF/MEK/ERK signaling pathway by vemurafenib[32]. Resistance to vemurafenib was later found to be caused by feedback activation of EGFR when BRAF is inhibited[33]. This finding has led to ongoing clinical trials that investigate combinations of inhibitors of BRAF, EGFR, and MEK with or without chemotherapy[34].

TP53 GENE MUTATIONS

TP53 gene is a very important tumor suppressor which

plays a role as a central regulator of different cellular processes including growth arrest and apoptosis, DNA damage, responses to stress, oxidative stress and aberrant proliferative signals[35]. *TP53* stops cell cycle in damaged cells until alteration is properly repaired, otherwise it triggers the apoptosis cascade in those damaged cells.

TP53 protein dysfunction is one of the common hallmarks of human solid tumors which has been reported in more than 25% of adenomas, and in 50%-70% of patients with CRCs. TP53 dysfunction is also playing a critical role in the adenoma to carcinoma transition[36]. The majority (about 80%) of TP53 gene mutations are missense mutations leading to the synthesis of a dysfunctional protein with an abnormally long half-life. Those missense mutations mainly occur within five hotspot codons (175, 245, 248, 273, and 282)[37]. Different *TP53* mutations are also reported in more than half of sporadic CRC patients[38].

APC/β-CATENIN MUTATIONS

Genetic disruption of APC leading to the activation of Wnt pathway, is one of the important early genetic event in colorectal tumorigenesis[39]. The *APC* gene product is a large protein that regulates development, chromosomal segregation, cellular differentiation, polarity, adhesion, migration, and also apoptosis. The APC protein is interacting with glycogen synthase kinase-3β (*GSK-3β*) and β-catenin (an important molecule of the Wnt pathway). Germline APC mutations are the cause for FAP (familial adenomatous polyposis)[40]. Somatic mutations in the APC gene are observed in 30%-70% of sporadic adenomas, and in around 70% of sporadic tumors[41]. These somatic mutations are mainly found between codons 1286 to 1513 (known as the mutation cluster region), while germline mutations are distributed throughout the entire gene[42]. As an alternative mechanism for APC gene dysfunction, APC promoter hypermethylation is also reported in more than 18% of primary colorectal carcinomas and adenomas[43].

DNA METHYLATION

The most widely studied epigenetic alteration in cancer is aberrant DNA methylation. DNA methylation is one of the important epigenetic modifications that can regulate gene expression. In humans, DNA methylation occurs at cytosine residues that precede guanines, called CpG dinucleotides (C-phosphodiester-G)[44,45].

Recently, along with a growing understanding of cellular epigenetic mechanisms, the role of pathologic epigenetics changes in human cancer became more visible[46]. It is becoming more evident that epigenetic events might be central to the cancer initiation and progression[44,45,47-52].

ABERRANT DNA HYPERMETHYLATION IN CRC

Hypermethylation of promoter regions leading to gene silencing, is frequently found in different types of human cancers[45,50]. Recently a third class of CRCs characterized by a high frequency of DNA hypermethylation has been reported. These cancers have been defined as having a "CpG island methylator phenotype (CIMP)". Weisenberger *et al*[53] could show CIMP in CRC, is based on the methylation status of five genes (CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1). CIMP-positive tumors exhibit unique clinicopathological and molecular features, including a predilection for proximal location in the colon, poor and mucinous histology and the presence of frequent KRAS and BRAF mutations[54]. Approximately, 80% of sporadic CRCs with MSI-H harbor biallelic hypermethylated *MLH1* alleles[53]. The transition from normal mucosa to adenomatous polyp is marked by both genetic and epigenetic alterations, which dysregulate important molecular pathways[55]. These epigenetic alterations include hypermethylation of a variety of genes, such as SLC5A8, ITGA4, SFRP2, PTCH1, CDKN2A, HLTF, and MGMT, and some of these play a role in the initiation and progression of adenomas to CRC[56,57].

To date, a large number of hypermethylated genes including *APC*, *ATM*, *BMP3*, *CDKN2A*, *SFRP2*, *GATA4*, *GSTP1*, *HLTF*, *MLH1*, *MGMT*, *NDRG4*, *RASSF2A*, *SFRP2*, *TFPI2*, *VIM*, and *WIF1,* have been found in stool DNA assays for the early detection of CRC[58].

The *SEPT9* gene methylation assay has recently been developed as a novel blood-based test for CRC (Epi proColon 2.0, Epigenomics AG, Germany). Septins are a family of conserved GTP-binding proteins which are scaffolding proteins during compartmentalization and cell division[59]. In human, there are 13 known septin genes (*SEPT1* to *SEPT13*). All septins can form heteromeric complexes; within terminal position of these octamer protein family, *SEPT9* plays an important role in subunit stabilization and polymerization[60]. Therefore, abnormal *SEPT9* or no *SEPT9* may seriously affect cytokinesis. The crucial role of *SEPT9* in the septin complex may be a key factor in CRC carcinogenesis when the promoter region of the *SEPT9* gene is aberrantly hypermethylated and the transcription is compromised[61]. To date, several independent clinical trials have proved the aberrant *SEPT9* gene methylation as a specific biomarker for CRC early detection and screening.

GENOME-WIDE DNA HYPOMETHYLATION IN CRC

While DNA hypermethylation can silence tumor-suppressor genes, global DNA hypomethylation

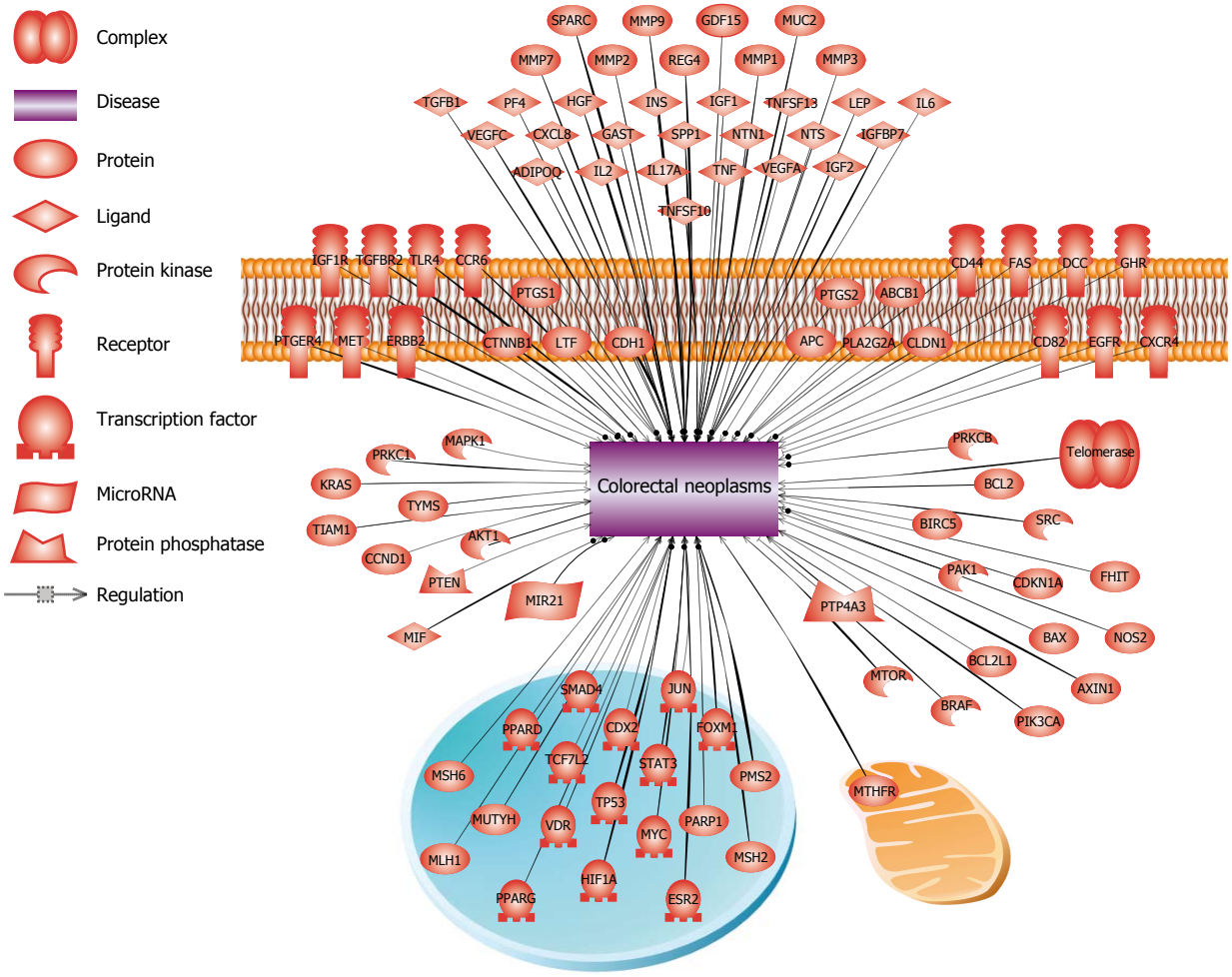

Figure 3 Genes associated to colorectal cancer development. Dysregulation of these genes as a single or in cooperation (due to the DNA mutation, epigenetic changes or as a consequence of change in the regulatory upstream genes/pathways), has been shown in different forms and stages of colorectal cancer.

can also influence CRC development by inducing chromosomal instability and global loss of imprinting[62]. Genome-wide hypomethylation occurs within repetitive transposable DNA elements, such as the long interspersed nuclear element-1 (LINE-1) or short interspersed nucleotide elements (SINE or Alu) sequences in many cancers, including CRC[63]. LINE-1 hypomethylation inversely associates with MSI-H phenotype and/or CIMP in colorectal cancer[63].

TUMOR SPECIFIC GENE EXPRESSION PATTERNS

Gene expression analyses of tumor derived cells/tissues in comparison to the normal tissue, reviled to a better understanding of interplay between dysregulated genes and the affected pathways in colorectal neoplasms. CRC is a very heterogenic disease and patients with even the same type of cancer, often have dissimilar genetic/epigenetic defects in their tumors which indicates why those patients respond heterogeneously to the anticancer agents. The use of DNA-microarray and next generation sequencing (NGS) technology have made it possible to assess the expression of tens of thousands genes in a single experiment[64]. These gene-expression signatures can be used for the molecular classification of different tumors as well as cancer subtypes[65]. Those genes which their dysregulation is linked to CRC are summarized in the Figure 3.

Several groups have reported different molecular classifications of CRCs using gene expression data from NGS or expression arrays and in some cases, these subtypes could provide predictive values[66,67] or prognostic information[68,69]. Validation of a 23-gene microarray-based prognostic signature showed around 70% relapse predictive value for CRC patients[70]. High throughput gene expression profiling reveals tumor subtypes that show overlap with previous classification systems using MSI, CIN, CIMP or KRAS/BRAF mutation status[66], which cannot be identified only based on single mutations or epigenetic alterations.

TUMOR SPECIFIC MICRORNA EXPRESSION PATTERNS

Abnormalities and dysregulations in non-coding regulatory RNAs can also contribute to tumorigenesis

and cancer development[71,72]. A class of small cellular RNAs, termed microRNAs (miRNAs), can lead to silencing of their cognate target genes. MicroRNAs are key players in regulating diverse cellular pathways.

It is known that some miRNAs can function either as tumor suppressors or oncogene[72,73], and expression profiling of microRNAs have revealed the characteristic signatures of these small regulatory RNAs in different cancers including colorectal cancer[71,74].

Recently, the interest for miRNA biomarker research in human cancer has increased due to the unique characteristics of miRNAs. First, miRNAs are remarkably stable under a variety of experimental and laboratory conditions. Second, due to their small size and the hairpin-loop structure, miRNAs are protected from RNase-mediated degradation, and thus are easily extractable from a wide variety of clinical specimens, including formalin-fixed paraffin embedded (FFPE) tissues, and a variety of body fluids including blood, saliva, urine, feces *etc*. Third, cell-free miRNAs are often protected from degradation because of being in high density lipoprotein particles, apoptotic bodies, microvesicles, and exosomes[75].

MiRNAs can have a critical role in invasion, migration and the progression of disease through epithelial mesenchymal transition (EMT) into metastases. Therefore, dysregulation of several miRNAs have been reported at different stages of CRC development and progress. More than 500 differentially expressed miRNAs have been associated with different stages of CRC. MiRNAs such as miR-20a, miR-21, miR-29a, miR-31, miR-34a, miR-92a, miR-200c, miR-215 and miR-375 were the most frequently dysregulated in CRC. Recent experimental analyses have validated a total of 530 miRNA-mRNA pairs in colorectal cancer, 200 unique miRNAs and 347 unique targets[76]. In *BRAF* mutated forms of CRC, a high expression of miR-31 was observed and suggested as a potential diagnostic/therapeutic biomarker[77]. A signature of miR-92a, miR-375 and miR-424 could discriminate invasive carcinoma from adenoma, representing a promising biomarker for the early diagnosis of CRC. The panel of six miRNA classifier (miR-21-5p, miR-20a-5p, miR-103a-3p, miR-106b-5p, miR-143-5p, and miR-215) has potential to distinguish between high and low risk of disease progression[76].

The functional role of miR-34a and miR-200 family members have been shown in metastatic form of CRC. In addition, miR-34a can be used as an independent predictor of recurrence among patients[78]. Overexpression of miR-622 was induced by radiotherapy in rectal cancers, causing poor response to the therapy[79]. In metastatic CRC patients with wild-type *KRAS* that responded to anti-EGFR therapy, profiling the panel of miR-99a, let-7c and miR-125b could have a diagnostic potential[80]. In the other hand, reduced expression of miR-181a was associated with poor clinical outcome in CRC patients treated with anti-EGFR[81]. Whereas, down regulation of the miR-7, a direct regulator of *EGFR*, was reported as a prognostic biomarker for tumors were resistant to targeted anti-EGFR therapy[82].

TELOMERE LENGTH DYNAMICS

Telomeres, the ends of chromosomes in mammals, are composed of a 6-bp variable repeat sequence (TTAGGG), which is added on by the telomerase and has a crucial role in maintenance of chromosomal stability[83]. Telomeric DNA (tDNA) is progressively lost during each cell division due to the end replication mispairing, oxidative damage or other end processing events[84,85].

Telomerase activity and telomere length have a crucial role in malignant transformation. In the early stage of tumorigenesis, losing the telomere sequences (telomere shortening) limits cell proliferation, and telomerase activation protects the ends of chromosome and suppresses tumorigenesis. While, in the late stages of tumorigenesis, telomere shortening triggers instability in the genome, and telomerase activation induces immortalization of cancer cells[86,87]. Figure 4 summarizes the genes that regulate telomere length and also telomerase activity. Recent data proposed that the severe genomic instability exist in telomere crisis, accelerates secondary genetic alterations that lead to carcinogenesis and indeed highlights the implication of pathologic telomere length changes in cancer pathways. Therefore, telomere length dynamics can serve as a useful indicator and biomarker in risk assessment and prediction of different stages of cancer[88-92]. Telomere dysfunction has been indicated as a negative prognostic marker in solid tumors[90,93,94].

Several studies demonstrated telomeres are shorter in CRCs compared to the adjacent normal mucosa. In addition, malignant tumors have relatively shorter telomere length than benign tumors[95-98]. Since tumors in sporadic form of CRC appear relatively later compared with the hereditary nonpolyposis colorectal cancer (HNPCC), also the number of divisions between initiation and clinical presentation in sporadic may be more than HNPCC, therefore their base line telomere lengths at initiation are likely to be shorter in sporadic form of CRC[2,90,99].

The evidence that telomeres were shorter in CRCs, even in well-differentiated tumors, suggesting the telomere length shortening as an initial event in colorectal cancer which directly reflects pathologic cell proliferation[100,101].

ANGIOGENESIS BIOMARKERS

Angiogenesis, critical step in cancer progression, is a new blood vessels generation from endothelial precursor, which is mediated through a group of ligands and receptors that work together[102]. A group of glycoproteins, including the placental growth factor (PIGF) and VEGF family (VEGF-A, VEGF-B, VEGF-C,

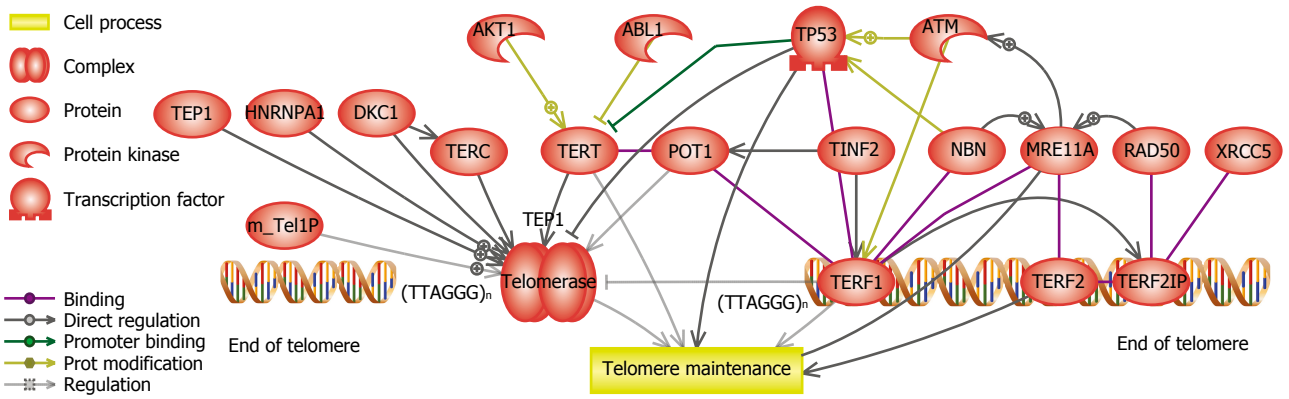

Figure 4 Genes which are involved in the telomere maintenance and telomerase activity pathway. Any dysregulation in those genes will lead to the pathologic telomere length dynamics during colorectal cancer development stages.

and VEGF-D), act as effectors of angiogenesis[102,103]. Several other factors including the PIGF, fibroblast growth factor (FGF), VEGF-C, VEGF-D, angiopoietin, hypoxia-inducible factor (HIF)-1α and HIF-2α, integrin, and platelet-derived growth factor, can overlap with the angiogenesis pathway, which makes the activation or inhibition outcomes very challenging[102].

The potential value for VEGF as a prediction or prognosis biomarker for metastatic CRC has been reported[104]. It is shown that the VEGF-1154G>A and VEGF405C>1 polymorphisms were associated with improved overall survival. This finding suggested that germline variants in VEGF-dependent and -independent angiogenesis can predict survival and treatment response to the first-line bevacizumab and oxaliplatin-based chemotherapy in patients with mCRC[105]. The importance of baseline levels of VEGF and soluble VEGFR-2 (sVEGFR-2) as predictive/prognostic biomarkers was shown in two independent phase-III studies evaluating the role of cediranib, an experimental angiogenesis inhibitor, in mCRC[106].

INFLAMMATORY BIOMARKERS

In patients with the inflammatory bowel diseases, chronic inflammation was suggested as a predisposing factor to CRC and the risk for developing CRC increases with longer duration of colitis. Therefore, anti-inflammatory therapies, such as 5-aminosalicylates, can significantly reduce the development of colorectal neoplasia in those patients[107].

C-reactive protein (CRP) is a very sensitive but non-specific systemic marker of inflammation. CRP is mainly produced in the liver in response to cytokines released during infection, trauma, surgery, burns, tissue infarction, advanced cancer, and chronic inflammatory conditions[108]. According to a recent study, the concentrations of CRP, might have a positive correlation to the CRC risk[109].

Interleukin-6 (IL6) secreted by the hematopoietic or non-hematopoietic cells, is a multifunctional cytokine and has a pro-inflammatory function *via* binding to a soluble IL6 receptor (sIL6R) or by acting on a transmembrane type 1 cytokine receptor[110]. IL6 upregulates several acute-phase proteins such as CRP, α1-antitrypsin, fibrinogen and serum amyloid A[111]. It is shown that IL6 is significantly augmented at the colonic tumor microenvironment[112].

TNFα is another pleiotropic cytokine which is secreted in response to any tissue damage or infection; some reports suggesting the role of TNFα in the pathogenesis of IBD[113]. The other inflammatory biomarker, macrophage inhibitory cytokine 1, is reported to be positively associated with CRC risk[114]. Certain inflammatory biomarkers from the CRC or tumor immune reaction, may lead to generation of individualized immune vaccines.

NON-INVASIVE BIOMARKERS

Blood biomarkers

As non-invasive biomarkers for the screening of colorectal cancer, particularly at early stage of the disease initiation, blood biomarkers could show very promising value. Theses blood-based markers could be also used to monitor therapeutic response in CRC patients as well as the detection of disease recurrence or relapse.

Circulating tumor cells: Circulating tumor cells (CTCs) are tumor cells (mainly cancer stem cells) derived from either primary tumors or metastases which are circulating in the peripheral blood. The presence of CTCs in blood is associated with progressive or metastatic disease. Therefore, can be used to monitor advanced stage disease in patients without other measurable surveillance markers.

The CTCs in colorectal cancer have epithelial origin with defined immunephenotype signature ($CD45^-$, $EpCAM^+$)[115]. Using the immunephenotype markers and sensitive cell sorting technologies, it is now possible to isolate and assess the complete genetic/epigenetic profiles of tumor derived CTCs. This holds promise for the development of more efficient

personalized treatment to eliminate cancer stem cells in CRC patients.

Circulating cell-free DNA: The discovery of circulating cell-free DNA (ccf-DNA) could open up a new possibility for non-invasive analysis of tumor derived genetic material, as it can be isolated from human body fluids[116]. The potential diagnostic/prognostic values of quantifying ccf-DNA in CRC patients compared to the healthy controls, have been assessed in different studies[117]. The ccf-DNA levels in those patients, could show significantly increase within the advanced disease stages and fluctuated during chemotherapy period[118].

Many studies have investigated MSI or LOH within ccf-DNA from the plasma/sera of CRC patients as very valuable non-invasive biomarkers[119]. Different studies have reported the diagnostic/prognostic value of mutated genes within ccf-DNA[120]. The detection of mutated *TP53* or *KRAS* genes in the ccf-DNA of CRC patients could predictive disease recurrence as well as the clinical outcome[121]. Aberrant DNA methylation can also have a diagnostic or prognostic value in the plasma/sera of CRC patients. Relying on aberrant DNA methylation markers, promoter methylation of several genes have reported as biomarkers for CRC including *ALX4*, *APC*, *CDKN2A/P16h*, *FRP2*, *MLH1*, *NGFR*, *NEUROG1*, *P16*, *RUNX3*, *SEPT9*, *TMEFF2, TP53* and *TPEF/HPP1*[122,123].

Circulating microRNAs: Due to the stability of circulating microRNAs as well as the potential role of the microRNA dysregulation at different stages of carcinogenesis, they have the potential to serve as very promising non-invasive biomarkers for different types of human cancers[124].

As a well-characterized oncogenic miRNA, miR-21 is considered one of the promising non-invasive biomarkers for the early detection of CRC. Dysregulation of miR-21 occurs frequently at early stages of the adenoma-carcinoma sequence; miR-21 is one of the most highly expressed miRNAs in CRC; and miR-21 is highly secreted by cancer cells, which can be measured in exosomes or as free miRNAs in plasma or serum[125].

As another potential circulating microRNA, the elevated serum level of miR-92a has been reported for the advanced adenoma and CRC[126]. There are also other studies showing the elevated circulating levels of miR-17-3p and miR-29a[127]. Significantly higher expression of miR-17-92 cluster and miR-135 were found in CRC patients in comparison to healthy controls which could discriminate CRC with an overall sensitivity and specificity of 74% and 79%[128].

Plasma levels of miR-92 could distinguish patients with CRC from gastric cancer, IBD and healthy control subjects[129]. A high diagnostic accuracy has been shown by a panel of miR-409-3p, miR-7, and miR-93 in discriminating CRC from controls with more than 90% sensitivity and specificity[130].

Circulating proteins: Shed or secreted proteins from different cancer cells into the bloodstream, can be detected by different methodological approaches like enzyme-linked immunosorbent assay (ELISA) or chromatographic and mass spectrometric (MS) technologies. Different protein-based biomarkers have been reported for CRC. These protein markers include circulating carcinoembryonales antigen (CEA), carbohydrate antigens (CA19-9, CA50, CA72-4), soluble Fas ligand (FasL), p53, and VEGF[131]. Of these markers, CEA could show a strong prognostic impact in CRC patients[132].

STOOL BIOMARKERS

The presence of tumor biomarkers in stool can be attributed to secretion, exfoliation or leakage[133]. Stool-based markers come from vital and apoptotic colonocytes, shed into the colorectal lumen. Since the stool markers are directly derived from the tumor cells, assumed to be highly specific biomarkers for CRC. These stool biomarkers include stool DNA (sDNA), which can be used for checking the MSI, aberrant DNA methylation or somatic mutation for specific cancer related genes, miRNAs, protein biomarkers as well as secretory molecules and biochemical materials resulted from metabolism of cancer cell. Considering the off-site testing and non-invasiveness together with the low potential costs, stool biomarkers gets more reappraisal. Recent advances in laboratory techniques, could introduce modern generation of stool tests with higher sensitivity and specificity rates for different subgroups of the colorectal cancer. These stool biomarkers should be very sensitive with higher specificity because positive test results lead to unnecessary, potentially morbid, and costly colonoscopies[134].

Human DNA is less than 0.01% of total DNA in stool and the vast majority (99.99%) of sDNA is derived from intestine bacterial or dietary; therefore, one of the important technical challenge of sDNA testing is specific detection of methylated or mutated human DNA within a pool of nontarget DNA[135,136]. Several panels of methylated genes within sDNA have been reported for different stages of CRC, involving *BMP3, CDH1*, *CDH13*, *CRBP1*, *CXCL12*, *ESR1*, *HLTF*, *ID4*, *IRF8*, *ITGA4*, *MINT1*, *MINT31*, *NDRG4, P14*, *P16*, *RUNX3*, *SFRP1*, *SFRP2*, *SLC5A8*, and *TIMP3*[137]. These panels were differing in the marker selection, assay methods, and patient populations studied which many of them could not be further validated in the bigger cohorts.

Although several stool-based tests (fecal immunochemical test (FIT), guaiac fecal occult blood test (gFOBT), immunological fecal occult blood test (iFOBT), and sDNA test) are clinically available for the detection of CRC, the sensitivity and specificity for tests is not sufficient due to many factors such as inconvenience

in sampling or misinterpretation. Recently, the US food and drug administration (FDA) has approved the Cologuard® test (Exact Sciences Corporation, United States). Cologuard® is a panel of multi-target sDNA test, which combines molecular tests for *KRAS* and β-catenin mutation, aberrantly methylated *BMP3* and *NDRG4* gene promoters and human hemoglobin immunochemical assay[138].

CONCLUSION

The fast growing knowledge of cancer biology, particularly in the field of genetic, epigenetic and molecular cell biology, could provide valuable objectives for the early detection of different malignancies including colorectal cancer. The poor outcome of advanced form of CRC, has prompted the need for reliable predictive and prognostic markers.

Circulating and stool-based tumor specific markers show promising potential as biomarkers. Additional biomarkers that have clinical applicability will continue to be proposed, tested, and developed from knowledge of the genetic and epigenetic changes in CRCs. This would facilitate more individualized treatment approaches, relying the specific molecular signature of tumors. Sequencing of individual cancer genomes that provide a comprehensive picture of driver mutations within a CRC, may become commonplace when costs for this technology are lower and rapid analytic systems are employed. This will be most beneficial with simultaneous development and use of therapies that can address the personalized findings.

Unfortunately, most of the identified biomarkers in different tumor studies, failed in the validation studies. The lack of consistency between biomarker panels within independent studies, highlights a major obstacle for the development of robust CRC biomarkers. For being able to use CRC associated biomarkers in clinical care of patients, large-scale studies are needed to identify optimal marker panels and validate those biomarkers in more cross-sectional and prospective cohort studies.

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