Molecular subtyping in colorectal cancer: A bridge to personalized therapy (Review)

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Abstract. Colorectal cancer (CRC) is a malignant tumor and a major cause of morbidity and mortality globally. The classic Tumor-Node-Metastasis staging system, which currently underlies the diagnosis and treatment of CRC, is primarily a 'one drug fits all' model for patients exhibiting the same pathological features. However, a high degree of variability has been established in the long-term survival outcomes of patients with CRC with similar pathological types and stages, which can be partially attributed to tumor-specific molecular biology to some extent. Molecular classification of CRC can further assist with understanding the biological behavior of tumor genesis, development and prognosis, and assist clinicians in improving or customizing the treatment strategy of CRC. In the present study, clinical studies carried out to date are reviewed, and their clinical value is discussed. A multilevel overview of the major molecular types of CRC is provided, in the hope that investigators are encouraged to combine multiple omics studies for interrogating cancer.

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers of the digestive system worldwide and can occur at any age. According to global Cancer Epidemic Statistics released in 2020 by the International Agency for Research on Cancer of the World Health Organization (GLOBOCAN2020) (1), an estimated 1,931,600 new cases and 935,200 deaths associated with CRC were reported globally, ranking third and second, respectively, in terms of morbidity and mortality. The classical Tumor-Node-Metastasis (TNM) staging system is still an important reference for clinicians to assess patient prognosis and provide individualized treatment (2,3); however, this staging method has limitations, with increasing evidence of significant differences in the prognosis of patients with CRC with the same stage and pathological type (4). In 1999, the National Cancer Institute of the United States proposed the concept of the molecular classification of tumors, using molecular characteristics through molecular analysis techniques (5). At present, the detection of KRAS, BRAF, microsatellite instability (MSI) and their mutations contributes to the clinical management of CRC and the selection of personalized drugs (6,7). The use of novel targeted and cytotoxic drugs has extended the median overall survival (OS) of patients with advanced CRC to 25-30 months (8). Despite its widespread use, the clinical translation of single molecular markers is not always consistent, which may be related to differences in data processing and algorithms applied to different patient cohorts, sample preparation methods and gene expression platforms. With the progress of molecular sequencing and gene molecular mechanism research, scientists have used a more organized and universal way of defining current disease patterns; the characterization of tumor biological characteristics, namely consensus molecular subtype (CMS) typing (9), has promoted cancer classification transition from 'mutation-centered' to 'transcriptome-based' molecular subtypes. CRC molecular typing plays an increasingly important role in the era of individualized precision medicine. Emphasis has been placed on classifying CRC based on genetic characteristics, tumor microenvironment and, more recently, immunological characteristics, with each classification system having its unique importance and clinical significance. The present review provides an overview of molecular typing and its clinical significance.

2. Genome-based molecular typing of CRC

Current evidence suggests that the occurrence and development of CRC is a multi-step, multi-stage and multi-gene process. It is widely considered to result from the interaction of environmental and genetic factors, as well as from the upregulation of tumor suppressor genes and proto-oncogenes. Based on the genetic mutations and the cytogenetic background of the genome, molecular typing based on the CRC genome is affected by the presence of the following: Chromosomal instability (CIN), microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and molecular markers.

CIN. CIN refers to the phenomenon of chromosomal variation in cells (10). It mainly consists of two parts: Chromosomal number variation, namely chromosomal aneuploidy, which is closely related to tumor deterioration, progression, metastasis and a poor prognosis (11-13), and abnormal chromosomal structure, such as recombination, ectopia, and inversion, among others (14). It has been shown that mutation accumulation of multiple proto-oncogenes, such as RAS, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α(PIK3CA), c-Myc, BRAF and tumor suppressor genes, such as adenomatous polyposis coli (APC) gene, tumor protein 53 (TP53), PTEN, deleted in Colorectal Cancer(DCC), can lead to CIN. Genomic instability promotes development of CRC. CIN tumors can develop through loss of heterozygosity (LOH) in chromosomes (14). Watanabe *et al* (15) classified CIN tumors into CIN-high (severe type; LOH ratio ≥75%), CIN-high (mild type; LOH ratio ≥33 and <75%), and CIN-low (LOH ratio <33%) according to the LOH ratio. Survival analysis showed that disease-free survival (DFS) and OS rates of patients with CIN-high tumors were significantly lower than those of patients with CIN-low tumors, corroborating that the CIN phenotype is an independent risk factor for CRC survival. CIN phenotype is most common in the distal colon (16).

CIN has been documented in most sporadic CRCs (Sp-CRCs) and tumors with APC germline mutations, with an APC mutation rate of only 1%. Nevertheless, little is known about whether CIN is an independent predictor of familial CRC. Some researchers concluded that although the sensitivity of CIN prediction for familial CRC was acceptable, it was not sufficient to be an independent predictor (10,17,18). One study found no significant difference in CIN between familial CRC cases and non-familial, control CRC cases (P=0.50) (18).

A substantial number of CRCs, known as interval CRCs (I-CRCs), are diagnosed in the period shortly after a negative colonoscopy result (i.e., no detectable polyps or CRC) and prior to the recommended follow-up screening (19). According to the American Cancer Society, ~5,200 Americans were diagnosed with an I-CRC in 2014, and nearly 2,000 succumbed to the disease (20). This particular type of CRC may be associated with genetic defects inducing genome instability, or may be a specific type of Sp-CRC. In response to this uncertainty, researchers performed a matching comparison experiment of I-CRC/Sp-CRC cases and found that CIN occurred in 80-85% of Sp-CRCs and I-CRCs, and the latter frequently exhibited gains and losses in chromosomes 8, 11 and 17 (20). One possible explanation is the inaccurate detection of certain polyps/tumors or similar clinical features leading to negative colonoscopies. Another explanation is that I-CRCs represent a distinct tumor subset with both CIN and MSI phenotypes, and that these two molecular features may play a synergistic role. Furthermore, the interval between colonoscopy and screening could also be an additional explanation.

The CIN phenotype tends to be more of a predictive tool in clinical practice, and patients with CIN-positive CRC have shown poor OS and progression-free survival outcomes, regardless of ethnic background, anatomical location and fluorouracil (5-FU) chemotherapy efficacy (21). Watanabe *et al* (15) retrospectively reviewed the expression of MSI and CIN in 1,103 patients and concluded that the CIN phenotype could be used as an independent risk factor for DFS and OS in stage II/III patients, and CIN-high could be used as a predictor of a poor prognosis. Only 8% of patients with CRC were either in stage I or IV. CIN is a driver of the metastasis of human cancer cells, which has been preliminarily verified in breast and lung cancer models (22); however, the progression pattern of the CIN phenotype in breast cancer and lung adenocarcinoma is not applicable in CRC. Orsetti *et al* (23) used array comparative genomic hybridization to analyze a group of 162 patients with CIN CRC, consisting of 131 primary cancer cases evenly distributed in stages I to IV, 31 metastases (28/31 formed a primary-tumor/matched-metastasis pair) and 14 adenomas. The results showed that the increased level of genomic instability represented by CIN was not entirely consistent with the progression from stage I to IV during the histopathological examination. In addition, with study of the molecular mechanism of CIN, the genetic variation or abnormal expression of some molecules that maintain chromosomal stability may become therapeutic targets and diagnostic markers (22,24-27). High levels of CIN are not conducive to the proliferation of tumor cells. It is widely considered that drugs could induce higher levels of CIN phenotype, leading to the spontaneous death of tumor cells. Heat shock protein 90 inhibitors may achieve this effect by inducing higher aneuploidy and limiting tumor cell growth (28). A phase II trial (29) showed that patients with CRC did not respond well to docetaxel (Taxotere®), which may be attributed to the fact that 85% of CRC tumors were of CIN type, and aneuploidy was less receptive to taxanes than diploid karyotype (21), associated with increased taxane resistance caused by abnormal spindle examination points in CIN (30).

MSI. Microsatellites are short nucleotide repeats (1-6 repeat units) that are heritable, unstable and highly polymorphic in the human genome (31). MSI refers to the change in the number of microsatellite tandem repeats within a certain location in certain cells. Importantly, if the DNA mismatch repair genes (MMR) show germline mutations or LOH, errors from microsatellite replication will be retained (MMR-Deficient (dMMR)/MSI) (32). MSI in CRC includes the majority of hereditary non-polyposis CRC (HNPCC) and 15% of Sp-CRCs (32,33). MSI commonly occurs in two situations (34): The first is the germline mutation of MMRs MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), (MSH6) or Postmeiotic segregation increased 2(PMS2), and the other is hypermethylation of the MLH1 gene promoter region, which are predominantly cases of Sp-CRC showing dMMR. According to the microsatellite expression, MSI can be divided into three

types: Microsatellite high instability (MSI-H), microsatellite low instability (MSI-L) and microsatellite stability (MSS). The two main techniques used to determine dMMR/MSI status include immunohistochemistry (IHC), designed to detect dMMR status, and molecular testing, which determines MSI status (35). Overwhelming evidence substantiates that MSI CRC often presents as poorly differentiated carcinoma and mucinous adenocarcinoma, mostly in the proximal colon with peritumoral lymphocyte infiltration (31,36-38).

The incidence in younger adults (patient younger than 50 years), early-onset CRC (EOCRC) is rising alarmingly. EOCRC is an important reflection of the younger trend of gastrointestinal tumors. Long-term tumor burden is becoming increasingly severe for patients with EOCRC. In previous studies, in younger patients, metastatic tumors represented an increasing proportion of all tumor stages (39). Compared with general patients with CRC, patients with EOCRC had a higher frequency of dMMR/MSI-H and a higher proportion of wild-type (WT) KRAS and BRAF, as well as a higher BRAF V600E mutation rate (40,41). Taken together, these results support changing the average-risk screening age from 50 to 45 years for all patients, with molecular characterization being an important breakthrough for clinical intervention. It is well established that patients with MSI-H CRC have a better prognosis and longer survival time than patients with either MSI-L or MSS CRC. Guastadisegni *et al* (42) analyzed the survival status of 12,782 patients with CRC and concluded that patients with MSI-H CRC had improved OS and DFS times. A meta-analysis involving >7,500 patients showed that MSI-positive tumors were superior to MSI-negative tumors in terms of MSI and survival assessment, suggesting that the genomic molecular marker status can be independently analyzed to assess prognosis (43). Current guidelines recommend harnessing MSI-H to guide CRC adjuvant therapy and improve the quality of individualized treatment. In this respect, according to the National Comprehensive Cancer Network® (NCCN) guidelines (44,45), patients with stage II MSI-H CRC may have an improved prognosis but may not benefit from 5-FU-assisted chemotherapy. Kim *et al* (46) performed MSI and MMR detection, and prognosis analysis on 135 patients who received FOLFOX-assisted chemotherapy (adjuvant oxaliplatin, 5-FU and leucovorin therapy) after radical resection of CRC. The results showed that DFS and OS times were not significantly prolonged in patients with MSI-H/MMR-deficient (MMR-D) CRC compared with patients with MSI-L/MMR-intact (MMR-I) CRC. It was not investigated whether patients with MSI-L/MMR-I CRC would benefit more from 5-FU chemotherapy. Guastadisegni *et al* (42) hypothesized that patients with MSS CRC would benefit more from 5-FU chemotherapy than patients with MSI-H CRC. In a retrospective study of 6,964 patients with stage II CRC (47), an attempt was made to determine the relationship between 5-FU-based adjuvant chemotherapy, primary tumor laterality, MSI status and OS. The results showed that for MSS-positive tumors, adjuvant chemotherapy was significantly associated with improved patient 5-year OS rate [hazard ratio (HR), 0.47; P<0.001], even in the absence of other risk characteristics. By contrast, there was no significant association between adjuvant chemotherapy and OS in patients with MSI-positive CRC (HR, 0.85; P=0.671). It is difficult to judge the sensitivity of patients to 5-FU based solely on MSI status, and multiple stable expression markers are needed for a comprehensive analysis. In recent years, immunotherapy has been increasingly used to treat MSI CRC (48,49). In 2017, the US Food and Drug Administration approved pembrolizumab to treat inoperable or metastatic dMMR/MSI-H solid tumors based on the high response rates observed in five clinical trials (50-55). Nivolumab was introduced in dMMR/MSI-H metastatic CRC (mCRC) in the same year (56). Frameshift peptides generated by frameshift mutations caused by MSI-H are highly immunogenic and respond well to programmed death receptor-1(PD1)/programmed death ligand 1 (PD-L1) inhibitors. In 2015, a study showed that the MSI status of tumors is closely related to the effect of immunotherapy (57). From later-line monotherapy (Keynote-164, CheckMate-142) and later-line dual-drug therapy (CheckMate-142), to first-line monotherapy (Keynote-177) and first-line dual-drug therapy (CheckMate-142), the role of immunotherapy in the treatment of dMMR/MSI-H CRC is expanding (53,56,58,59). The efficacy of nivolumab plus ipilimumab in the treatment of patients with advanced dMMR/MSI-H CRC is reflected in the 2021 NCCN guidelines and the 2022 American Society of Clinical Oncology (ASCO) conference report (60). Dual immunotherapy can effectively reduce the occurrence of drug resistance, while B2M or JAK1/2 gene mutations associated with resistance to traditional immunotherapy do not affect the benefit of MSI-H CRC to PD-1 antibodies (61); however, immunotherapy will not be effective for the treatment of MSI-H mucinous adenocarcinoma CRC. Due to the persistence or potential toxicity of immunotherapy, a balance between efficacy and toxicity is necessary. It is worth mentioning that since immune checkpoint inhibitors (ICIs) are not effective in MMR-proficient (pMMR)/MSS mCRC, MMR IHC and MSI testing should be performed prior to ICI initiation to minimize the chance of pMMR/MSS tumors being misdetected as dMMR/MSI (62,63). Lynch syndrome is an aggressive autosomal dominant genetic disorder with an ~80% lifetime risk of cancer recurrence caused by germline mutations in the MMR genes (MLH1, MSH2, MSH6 and PMS2) (64). The NCCN guidelines recommend MMR or MSI testing for Lynch syndrome in all patients with a history of CRC (44,45). Some researchers consider that the relationship between MSI and CIN is not independent, and that both can be expressed in one patient with CRC, namely a patient with MSI-positive/CIN-positive CRC, although this is rare (65). The frequency of MSI-positive/CIN-positive tumors was recorded as 12 (Sp-CRCs) and 14% (I-CRCs), respectively (19). Furthermore, ~25% of patients presented with MSI-negative/CIN-negative CRC (66-69). A study stratified survival by CIN and MSI status, and concluded that the univariate survival benefit of stage II and III CRC associated with MSI-positive status was not independent of CIN status during multivariable analysis (21,67). Future experiments designed for the three forms of genomic instability [CIN, MSI and CpG island methylator phenotype (CIMP)] may clarify the relationship between the three.

Mutations and genomic instability contribute to inter-tumor heterogeneity. The sub-clonal phenomenon that exists throughout tumor progression is called intra-tumor heterogeneity (ITH). It has been reported that ITH can be detected in

almost all cancer types and is associated with tumor prognosis and drug resistance (70-73). A typical example of ITH is the molecular differences between primary tumors and metastases, such as MMR pattern or MSI status. After assessing the MMR status of mCRC, fewer patients with mCRC showed heterogeneity of MMR status between the primary and corresponding metastatic sites (11.9 and 18.7%, respectively), among which patients with peritoneal metastasis tended to exhibit this feature. Furthermore, the prevalence of heterogeneous MMR phenotypes in primary tumors with dMMR was significantly higher than that with pMMR (P<0.001) (74,75). However, it is noteworthy that various factors, such as the expertise of the pathologists, the quality of the tumor tissue sampling and staining can contribute to these discrepancies.

CIMP. CpG islands are regions rich in cytosine (C) and guanine (G) dinucleotides in the gene, with CG content >50%, length >250-550 base pairs, and a CpG value of 0.6 or greater (76-78). It has been shown that the pathogenesis of CRC is related to DNA methylation, with hypomethylation of genes in non-promoter regions and hypermethylation of genes in promoter regions (79). DNA hypomethylation can lead to oncogene activation, gene marker deletion and chromosomal stability. Hypermethylation of promoter sequences interferes with the normal expression of tumor suppressor genes and DNA repair genes, which is known as epigenetic silencing (80). This hypermethylated phenotype is called CIMP. Various classification criteria have been developed to describe the tumor characteristics of CIMP, each with unique molecular and oncological characteristics, and no gold standard has been established. Weisenberger *et al* (81) divided CRC into CIMP-positive and CIMP-negative CRC, according to five gene combinations (CACNA1G, IGF2, NEUROG1, RUNX3 and SOCS1). Similarly, Ogino *et al* (82) classified CRC as CIMP-High (CIMP-H), CIMP-Low (CIMP-L) and CIMP-negative based on eight gene combinations (RUNX3, CACNA1G, IGF2, MLH1, NEUROG1, CRABP1, SOCS1, and CDKN2A). This classification has been confirmed to be genetically associated with TP53, KRAS, BRAF, MSI and specific histological types (poorly differentiated or mucinous) (83,84); however, the relationship with CIN remains unclear. An increasing body of evidence suggests that CIMP-type molecular pathways mostly occur in the proximal colon, mainly in elderly women (84-86). Moreover, it has been observed that the frequency of gene hypermethylation in normal colon mucosa in women and elderly patients with CRC is higher than that in men and young patients (86).

Different methods of CIMP identification and experimental populations may result in different pathological characteristics. Weisenberger *et al* (86) showed that the right colon is a high-risk site for CIMP, and this classification is associated with advanced age in women, with the hallmark mutation of BRAF (V600E), loss of hypermethylation of the MLH1 promoter and loss of TP53. There is ample evidence suggesting that CIMP is closely associated with prognosis, but it remains unclear whether this association is positive or negative. Ogino *et al* (87) showed that CIMP-H was an independent predictor of colon cancer-specific low mortality. During the stratified analysis, CIMP-H was associated with significantly reduced colon cancer-specific mortality regardless of MSI and BRAF status. However, a more recent meta-analysis (88) involving 15,315 patients with CRC confirmed that CIMP-H CRC was associated with poorer OS/DFS/PFS/RFS times than CIMP-L/negative CRC. Furthermore, a survival disadvantage was observed in terms of OS, especially in stage III-IV and pMMR tumors. In addition to its prognostic value, the role of CIMP in the prediction of the chemotherapy response is another issue requiring resolution. Much controversy surrounds the efficacy of 5-FU-based chemotherapy against CIMP-positive CRC. Cha *et al* (89) showed that CIMP was associated with adverse outcomes for patients receiving chemotherapy for mCRC. Jover *et al* (90) concluded that CIMP-positive patients did not significantly benefit from 5-FU-based adjuvant chemotherapy after following up 302 patients with CRC, while CIMP-negative patients receiving chemotherapy experienced significantly prolonged DFS times. Iacopetta *et al* (91) reported contrasting findings that patients with CIMP-positive CRC can benefit from 5-FU treatment, mainly related to the association between CIMP positivity and intracellular folic acid metabolism, and gene silencing caused by DNA methylation. A recent study claimed that CIMP-positive tumors are potentially more responsive to the topoisomerase-inhibitor, irinotecan (92). Although CIMP has been reported as a potential prognostic biomarker for drug decision-making, overall research on treating CIMP-positive tumors with hypomethylating drugs appears to be limited. Indeed, the lack of a widely accepted CIMP phenotype and the correct stratification of patients with CRC according to the CIMP status are key issues for future CRC trials. Since CIMP was shown to be a tissue-specific phenomenon, DNA methylation information obtained only by array probes or other low-density techniques is far from sufficient, and new analysis methods, such as PacBio single-molecule real-time sequencing or nanopore sequencing are needed (93). Assuming that the CIMP pattern is stable across tumor sections and that epigenetic drug delivery and protection against side effects are improved, optimal antitumor activity is expected for CIMP-positive tumors.

Molecular markers. Some patients benefit from the wide application of molecular targeted therapy, and identifying molecular markers is an important prerequisite for screening patients with CRC who can benefit from targeted drugs.

RAS. The RAS gene is the most common proto-oncogene in human tumors. RAS can encode a group of small molecular proteins homologous to G proteins, called RAS proteins. When a RAS protein is mutated, it cannot normally complete its signal-mediated transduction process, and abnormal cell growth, differentiation and material transport occur, leading to uncontrolled proliferation and carcinogenesis. Importantly, the KRAS mutation rate in CRC is 30-50% (94,95). Sugimoto *et al* (96) hypothesized that as a precancerous lesion of CRC, the progression of laterally spreading tumor-granular was closely associated with RAS gene mutations, with RAS mutation rates up to 54.1%. The predictive significance of RAS mutant (mt) on the anti-EGFR drug response rate and survival time in patients with mCRC has been confirmed in previous studies (97-100). According to the NCCN guidelines, all patients with mCRC should be tested for the genotype of RAS (KRAS, NRAS) and BRAF mutations in

tumor tissue (6,7). After resection of metastases, a negative association between RAS mutations and patient survival has previously been reported (101). Importantly, patients with any known KRAS (exon 2 or non-exon 2) or NRAS mutation should not be treated with cetuximab or panitumumab, while other targeted therapies, such as bevacizumab, can still be used. It has been established that patients with mCRC carrying WT RAS can benefit from anti-EGFR therapy with prolonged OS and PFS times (102-104). In an analysis of patients with CRC of stage III MSS who received FOLFOX chemotherapy plus or minus cetuximab, KRAS was used as a marker of a poor prognosis (105). The RAS status was also included in the CMS classification established in 2015 (9). CMS analysis of patients with mCRC KRAS (exon 2 WT) in a previous FIRE-3 study showed that the CMS3 and CMS4 subgroups responded significantly better to cetuximab than to bevacizumab (106).

BRAF. BRAF is an important transduction factor in the EGFR signaling pathways of RAS, RAF, MEK, MRK and MAPK, and regulates various physiological processes of cell growth, differentiation and apoptosis. The mutation rate of BRAF in CRC is ~10% (107), and BRAF mutations are associated with the proximal colon and MSI (81,94,108). BRAF mt is often associated with a poor prognosis (109-111). A study demonstrated that patients with CRC in BRAF mt stage III have a higher risk of recurrence (112). By contrast, a study by Birgisson *et al* (113) reported that patients with CRC with both MSI and BRAF (V600E) mutations had a low recurrence rate, while researchers observed significantly higher recurrence rates in patients with MSI and KRAS mutations. Consistently, Seppälä *et al* (34) showed that in patients with stage I-II CRC, BRAF (V600E) mutation in conjunction with MSS is negatively associated with quality of life, and the prognostic potential of MSI negates the harmful effects of BRAF (V600E) and presents a positive prognosis. The IHC assay found that MLH1 expression was lost, but BRAF (V600E) was present, which excluded Lynch syndrome. This finding may be attributed to patients with MLH1 promoter methylation generally having BRAF mutations, and BRAF mutations almost always occur at a single site, V600E. BRAF mutations generally occur in patients with Sp-CRC, but not in patients with Lynch syndrome. The NCCN guidelines recommend that patients with mCRC should be tested for BRAF in tumor tissue (6,7). For patients with mCRC and RAS WT/BRAF mt, the guidelines do not recommend treatment with anti-EGFR monotherapy or in combination with chemotherapy agents.

The above two markers (RAS and BRAF) have been investigated in depth in previous studies, and PIK3CA and HER2 have also attracted the attention of clinicians. Current evidence suggests that the mutation rate of PIK3CA is 15-20% (114). In 2004, researchers reported a high frequency of PIK3CA mutations in human cancer cells such as breast cancer (frequency 7.1-35.5%), colorectal cancer (16.9-30.6%), ovarian cancer (33%), lung cancer (0.6-20%), among others, and subsequent studies identified PIK3CA as a risk factor for numerous types of cancer, including CRC (115). Currently, the American Society for Clinical Pathology, College of American Pathologists, Association for Molecular Pathology and ASCO guidelines (116) on the evaluation of molecular biomarkers of CRC do not recommend routine PIK3CA testing for treatment outside of clinical trials. *In vitro* cell and animal experiments have shown that some classical PIK3CA inhibitors, such as wortmannin, LY294002 and rapamycin, exhibit anti-tumor growth; however, the serious toxic side effects and drug resistance have limited further clinical trials (117,118). In addition, tumor cells treated with PI3KCA inhibitors tend to stop growing rather than undergo apoptosis, allowing tumor cells to develop drug resistance in various ways. It was shown that patients with PIK3CA-mutant CRC had a poor prognosis (119). An increasing body of evidence suggests that the benefit of aspirin in controlling overall CRC mortality may be more significant in PIK3CA-mutated CRC (114,120). HER2 is a proto-oncogene encoding a 185-kDa plasma membrane-bound tyrosine kinase receptor, a member of the EGFR gene family. HER2 amplification/upregulated expression can be detected in 3-5% of patients with RAS WT mCRC, mutually exclusive to KRAS, NRAS and BRAF mutations, and highly consistent between primary and metastatic tumors (121). HER2 has long been considered a marker of a poor prognosis, but multiple previous studies have shown that HER2 positivity is not significantly associated with prognosis (122-124). Accordingly, no consensus has been reached on its predictive effect. In patients with WT RAS, HER2-positive mCRC, the NCCN guidelines recommend trastuzumab combined with lapatinib/pertuzumab (6,7). In addition, HER2 amplification has been reported as one of the causes of patient resistance to EGFR therapeutics, and HER2 may serve as a negative predictor of anti-EGFR therapy (125). Therefore, anti-HER2 therapy may be a more reasonable option for patients with mCRC tested for HER2 upregulated expression before treatment with cetuximab and panitumumab. More recently, trastuzumab deruxtecan (DS-8201) has shown promising and long-lasting activity in patients with refractory, HER2-positive mCRC, including patients previously treated with HER2-targeted therapy, as preliminarily confirmed in the phase II trial DESTINY-CRC01 (126).

In most cases, a single marker characterized by mutations is insufficient to explain the heterogeneity in patients with CRC. In an attempt to refine the molecular map, combinations of multiple markers have the opportunity to overcome this limitation. One study (127) attempted to combine BRAF, PIK3CA and RAS testing and increased the proportion of patients benefiting from anti-EGFR treatment from 36.1 to 41.2%. The selection of drugs targeting the altered multiple gene targets in the MAPK pathway is expected to reduce drug resistance and improve the response rate. Besides, molecular classification using these biomarkers can be used to classify patients. For example, Gil-Raga *et al* (128) used BRAF (V600E), RAS and MMR status to divide 105 cases of stage I-III CRC into five molecular subtypes to identify differences in prognosis. In addition, it is widely thought that CRC is an umbrella diagnosis encompassing numerous rare disease subtypes, in a context where the complexity of molecular markers is increasing, and the combination of different markers emphasizes the significance of comprehensive genetic testing (125,129).

The fundamental significance of molecular typing is to better guide CRC-targeted therapy, prolong DFS, and improve patient prognosis and quality of life. Discussion of Molecular typing alone is less comprehensive, and individualized assessment of disease conditions inevitably takes into account demographic characteristics, clinicopathological characteristics, molecular markers, lifestyle and nutritional factors, and

chemical agents. Subsequent classification reports, such as the Jass Classification of CRC (130), CCS Classification (131), Ogino Classification System (132) and Mangi Classification of CRC (133), mostly used CIMP, MSI, CIN, RAS and other markers as prototypes to explore the future direction of neoadjuvant therapy and targeted therapy in the clinical environment.

3. Transcriptome-based molecular typing of CRC

Due to the heterogeneity of tumors, patients often exhibit strong differences in response to classical treatment regimens, resulting in a weak response, poor tolerance and even death. Previously established classification methods, such as Jass Classification, Ogino Classification, Mangi Classification and CCS Classification, may be appropriate for a certain group of individuals since they were based on studies with heterogeneous inclusion and exclusion criteria, tumor type, classification and mechanism (134). In previous years, with the development of sequencing technologies, Guinney *et al* (9) optimized data processing, algorithm bias, sample preparation method and classification basis, standardized the differences in queue selection, and analyzed genetic data and tumor types from multiple platforms. Finally, a CMS typing method was proposed. This classification method is currently recognized as the most robust classification method, mainly based on tumor biology rather than clinical results, since it can easily capture the inherent biomolecular heterogeneity of CRC and is expected to become the basis of immunotherapy or targeted therapy. There are 5 subtypes (CMS1, CMS2, CMS3, CMS4 and mixed type) with distinct molecular properties and clinical characteristics. CMS1 (microsatellite instability immune, approximately 14% of CRCs) CRC is hypermutated and associated with both MSI and strong immune activation. CMS2 (canonical, approximately 37% of CRCs) CRC displays epithelial differentiation and strong upregulation of Wnt and Myc downstream targets, both of which are classic pathways for the development and progression of CRC. CMS3 (metabolic, approximately 13% of CRCs) CRC is associated with epithelial and marked metabolic abnormalities. The enrichment of multiple metabolic characteristics in epithelial CRC cells is consistent with the occurrence of KRAS-activated mutations, which have been described as inducing significant metabolic adaptations. In CMS4 (mesenchymal, approximately 23% of CRCs) CRC, transforming growth factor-β (TGF-β) is activated with enhanced interstitial invasion and angiogenesis. Finally, mixed type (approximately 13% of CRCs) CRC represents a transitional phenotype or intratumoral heterogeneity (9). The main hallmarks of CMS are briefly described in Fig. 1.

CMS can be used for prognostic evaluation. TenHoorn *et al* (135) summarized the clinical predictive significance of CMS in a meta-analysis and showed that for patients with mCRC, a CMS2-4 tumor was associated with a better survival rate than a CMS1 tumor, and that a CMS2 tumor had the most favorable prognosis. By contrast, for patients with localized CRC, a CMS4 tumor was associated with worse prognostic quality [OS, Relapse-free survival (RFS) and Survival after relapse (SAR)] compared with a CMS1-2 tumor. Researchers analyzed the clinicopathological data of 4,151 patients with CRC, and the results showed that CMS4 was associated with the shortest OS time, while CMS2 was associated with the longest OS time. Even if patients with CMS2 experienced recurrence, the survival rate post-recurrence remained significantly superior (136). Over the years, significant inroads have been achieved in CMS exploration for precision therapy. There is ample evidence suggesting that in local stage II and III CRC tumors, adjuvant chemotherapy could increase the OS time of CMS2 and CMS3 patients compared with surgery alone (137-139); however, the benefit was not significant for CMS1 and CMS4 (135,140). The differences in the therapeutic efficacy of CMS2 and CMS3 versus CMS1 and CMS4 may be due to intrinsic molecular differences among epithelial, mesenchymal and immunogenic tumors (135). Several studies on mCRC have consistently pointed to the benefit of irinotecan-based chemotherapy regimens in increasing CMS4 OS and RFS rates (141-144). In particular, most peritoneal metastases are of the CMS4 type, and a meta-report suggested that a combination of cetuximab or bevacizumab and irinotecan could be considered as a first-line treatment for CMS4-type mCRC (135). Notably, the prediction and prognostic value of CMS classification on the treatment benefit and prognosis of mCRC have been extensively studied. It was found that CMS classification was associated with PFS and OS in patients with mCRC, and patients with CMS2 and CMS3 were more likely to benefit from bevacizumab combined with capecitabine, while patients with CMS4 had more robust survival benefits following treatment with bevacizumab plus FOLFIRI (135,142,145). In addition, numerous clinical trials have shown that CMS types are associated with survival status after the use of different chemotherapy and targeted drugs. Two drug trials [FIRE-3 (106) and CALGB/SWOG80405 (146)] found differences in response rates for cetuximab in combination with irinotecan or oxaliplatin in tumors with different CMS types, which can be attributed to the fact that irinotecan and cetuximab work synergistically in patients with CRC with all CMS types; however, the lack of tumor fibroblasts in the CMS2 and CMS3 internal environment was conducive to the synergistic effect of oxaliplatin and cetuximab. Despite CMS1 and CMS4 being rich in tumor fibroblasts, and the ability of oxaliplatin to activate this internal environment to release cytokines antagonizing the effectiveness of cetuximab, treatment efficacy is poor (147). In addition, it has been shown that cetuximab is more beneficial for WT KRAS CMS2 patients (131). Besides, patients with CMS4 receiving cetuximab plus FOLFIRI (5-flurouracil, leucovorin, irinotecan) were associated with superior OS and PFS rates compared with those receiving bevacizumab plus FOLFIRI, whereas CMS1 patients were more receptive to bevacizumab (106). Moreover, FOLFOX combined with bevacizumab instead of cetuximab was associated with improved OS and PFS rates in patients with CMS1 (146). Immunotherapy has gained significant momentum over the past years. M7824 is an anti-PD-L1/TGF-β trap fusion protein that inhibits TGF-β and PD-L1 pathways to exert antitumor effects (148). If CMS typing is used as an overall biomarker for patients with mCRC, cases with CMS4 type do not benefit from this drug, while CMS1 type tumors are more likely to benefit from immunotherapy (149).

CMS includes numerous significant genetic and biological indicators, and is human-centered to predict survival and guide drug use. The selection of samples (left half, right half or rectum), the selection of multiple gene states or indicators,

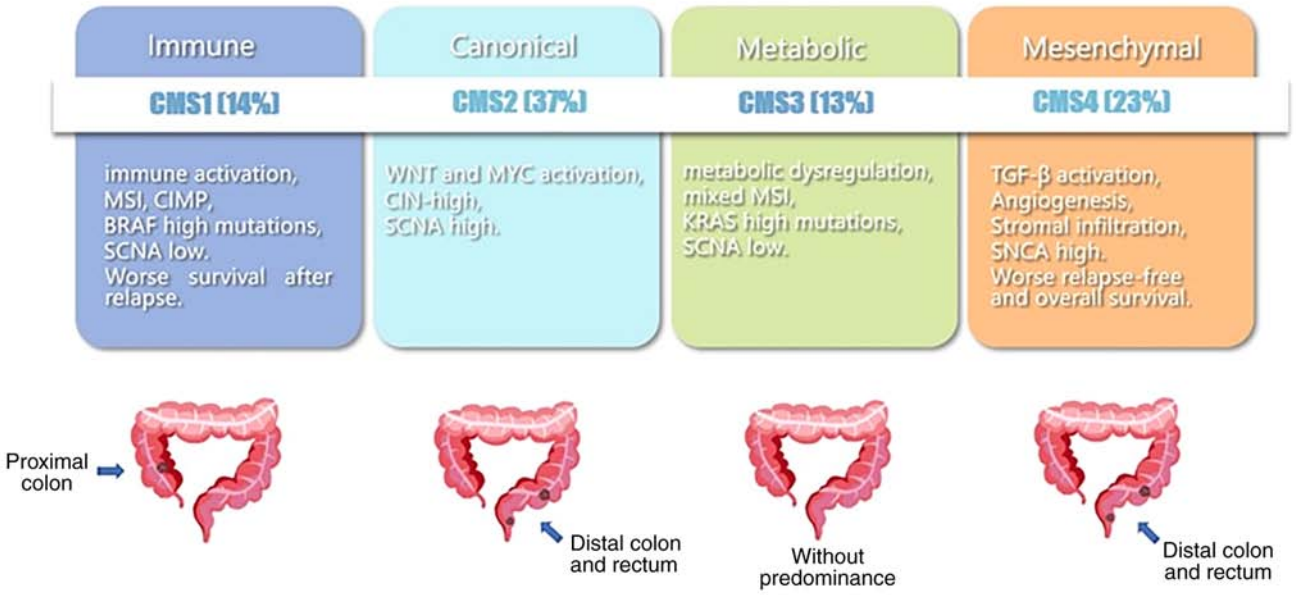

Figure 1. Main characteristics of CMS classification in colorectal cancer. CMS, consensus molecular subtypes; CIMP, CpG island methylator phenotype; MSI, microsatellite instability; SCNA, somatic copy number alterations; TGF-β, transforming growth factor β.

method of detection, and how to conduct classification analysis are still issues to be considered in prospective studies. There is a long way to go before CMS can be truly brought into clinical practice.

4. Proteome-based molecular typing of CRC

Using proteomics to study CRC, multiple proteins and their interactions can simultaneously be studied to reveal the biological mechanisms that occur at the protein level in the pathological environment of cells. Compared with genomics and transcriptomics, protein, as the main executor of human life activities, more directly reflect the biological phenomena. Protein expression is dynamic and diverse (150,151). Often, changes in abundance or function are not parallel to gene changes. Given the existence of multi-level regulation from the mRNA to the protein level, the transcription level cannot fully reflect protein expression level (152,153). Importantly, proteomic analysis enables observation of the biological behavior of cells directly, which is the basis of establishing proteomic molecular typing. Notably, Zhang *et al* (154) performed consistent clustering of proteomics data of 95 CRC tissue specimens, and divided CRC into five categories, A-E, describing the correlation between CRC and genome. Categories A, D and E were closely related to CIN, while B and C were closely related to MSI. More recently, Li *et al* (155) analyzed the clinical tissues of 146 patients with CRC, including 70 patients with mCRC, and divided CRC into three subtypes (CC1, CC2 and CC3) at the proteomic level. The three subtypes were associated with different clinical prognoses and molecular characteristics. For example, patients with CC3 CRC have a worse prognosis than CC1/2 patients, similar to mCRC. In addition, the results were consistent with those of CMS. Further phosphoproteomic profiling identified cases with mCRC. In addition, multi-omics collaboration and appropriate drug sensitivity tests are expected to identify an accurate target for drug selection for specific tumor types. The discovery of unique molecular markers and effective drug targets is still expected to integrate proteome and phosphorylated proteome analysis, and systematic validation in large tumor cohorts, and needs to be compared with established diagnostic methods to select the best or optimize the current diagnostic markers, which is the limit of their clinical translation (155,156). In addition, proteomics research has limitations such as complex preparation, inconvenient storage, difficult data analysis and poor repeatability, making it difficult for this method to become the mainstream method of diagnosis or medication guidance.

5. Conclusions

The significance of CRC molecular typing lies in predicting survival, customizing precision treatment strategies and predicting drug resistance. Although exciting progress has been made in the molecular typing of CRC, the clinical transformation of molecular typing, especially CMS typing, still faces great challenges and opportunities. At present, there is no unified system for the molecular typing of CRC, and there are crossovers among various subtypes. Therefore, it is essential to form a unified molecular typing by integrating the advantages of numerous molecular typing methods and summarizing the similarities and differences of various subgroups of CRC. Moreover, there is a lack of clinical detection methods, and the current mainstream molecular typing method is not applicable during clinical practice due to its lack of convenience and economical costs. Moreover, the process of CMS diagnosis by clinicians and pathologists is cumbersome, with a dearth of specific biomarkers to determine CMS, which fails to achieve the effect of strong practicability, easy availability and high accuracy of prediction shown by HER2, estrogen receptor and progesterone receptor in breast cancer management (157). Screening for fairly robust biomarkers is a multi-stage process

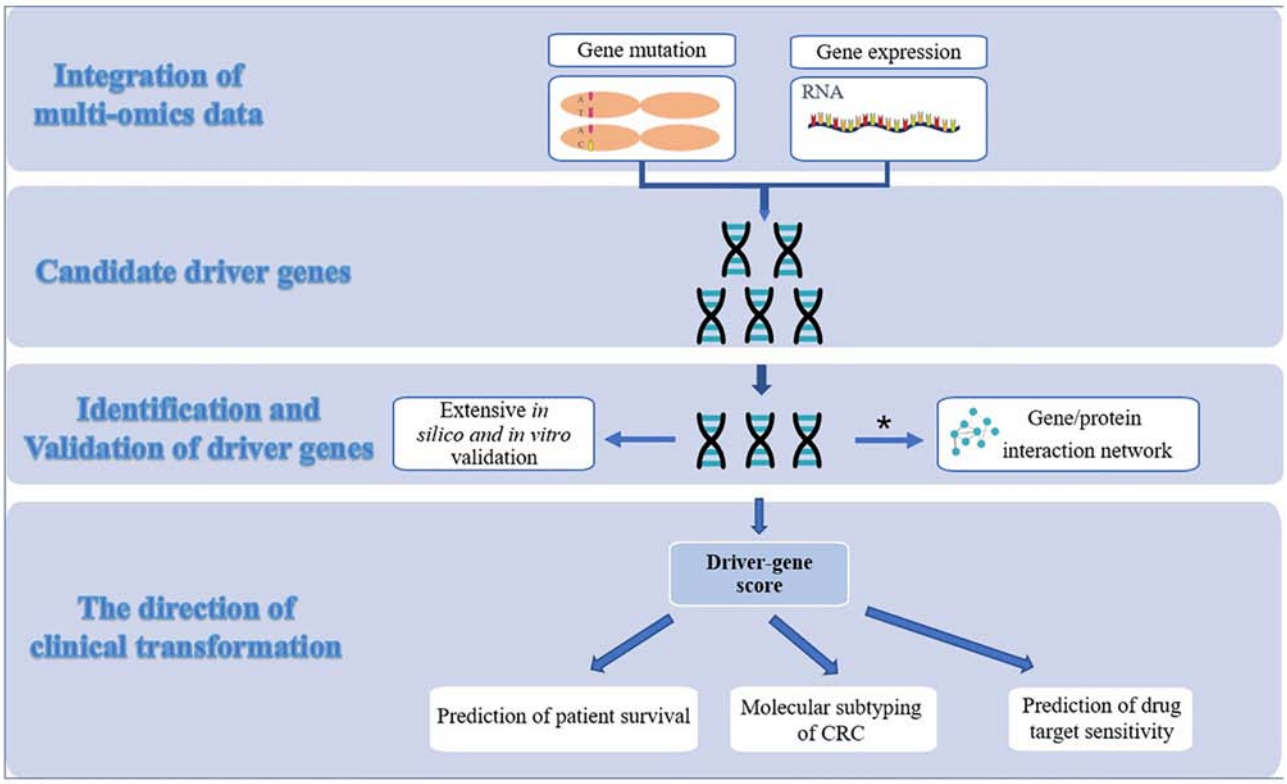

Figure 2. Network analysis model integrating multi-omics. The model framework or analysis pipeline is constructed to integrate gene mutation, expression and data function in multiple dimensions and multi-omics, identify cancer-related driver genes (even allowing identification of patient-specific driver genes), further develop gene function scores, and subsequently improve clinical validation (including survival prediction, discovery of new molecular typing and drug target sensitivity prediction). The asterisk represents that the final identified driver genes are associated with the gene/protein interaction network, and changes in driver gene status can affect the function of the linked genes and proteins.

involving biomarker discovery, model development, laboratory validation and prospective study validation. Besides, the current classifiers and gene maps should be optimized and simplified to find the most advantageous predictive genes in the list of characteristic genes. For example, based on the CCS typing of CRC, five markers (CDX2, FRMD6, HTR2B, ZEB1 and KER) were selected from a total of 146 characteristic genes in the CCS classifier, and were subjected to IHC staining and further classified in combination with MSI status (158). The accuracy of molecular diagnostic tests largely depends on the quality of paraffin-embedded tissues and the identification of tumor-rich regions, which pose an additional task for pathologists (159,160).

Single omics data can only provide a limited insight into the intrinsic molecular characteristics of CRC, whereas integration of multiple omics offers a more comprehensive approach to understanding tumor heterogeneity, uncovering the intricate regulatory pathways of the disease and ultimately improving the accuracy of CRC classification (157,161-165). The Cancer Genome Atlas has multi-omics data from >10,000 patient samples across 33 types of tumors, providing a comprehensive data set to explore the origin and progression of tumors from multiple angles (166,167). This presents both opportunities and challenges for the integration of multi-omics analysis. A comprehensive analysis of muscle-infiltrating bladder cancer showed that multi-omics classification was more effective than mRNA-based clustering analysis for guiding clinical decision-making (157,168). Banias *et al* (159) described three molecular subtypes of CRC (epithelial, mesenchymal and mixed) based on the IHC markers E-cadherin, β-catenin, maspin and vimentin. A novel relationship between intracellular maspin expression and MSI, tumor budding and prognosis has far-reaching clinical significance. Integrative approaches through network-based analysis have been previously developed to predict the functional impact of driver gene mutations (169-171) (Fig. 2). Successful transformation of routine clinical practice in molecular typing still requires multidisciplinary and multi-omics collaboration between basic cancer research, bioinformatics and clinical research.

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Availability of data and materials

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Authors' contributions

CLW, HZ and YXL reviewed the literature and collated the relevant data. HZ and YXL made substantial contributions to

conception and design of the present study and participated in the collection and interpretation of relevant data. CLW and HQH designed the article structure. CLW and GYW wrote the original draft. HQH, YLW and GYW reviewed and edited the manuscript. All authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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