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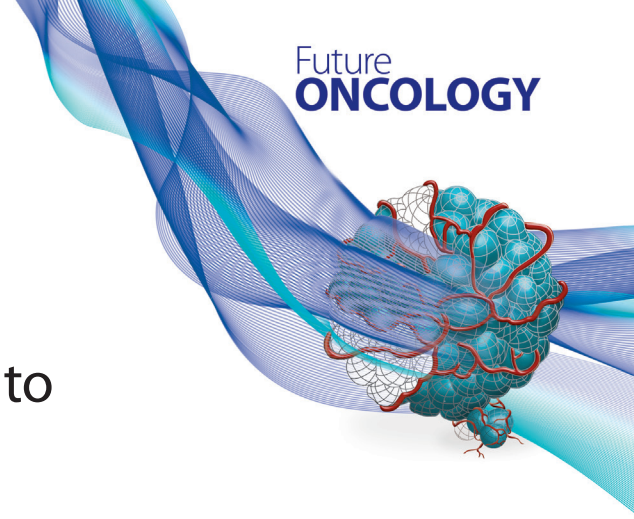

The colorectal cancer immune microenvironment and approach to immunotherapies

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Colorectal cancer (CRC) is the third most common cancer in the world, with about 1.4 million cases diagnosed worldwide in 2012 [1]. The prognosis for CRC patients is largely dependent on the stage of the tumor at diagnosis. In the USA, the 5-year survival rates following surgical removal of tumors for localized (stage I), regional (stages II and III) and distant (stage IV) cases are 91.1, 71.7 and 13.3%, respectively [2]. Current options for standard treatment of CRC include surgical removal alone for stage I and for most of stage II CRCs and surgical removal followed by adjuvant 5-fluoruracil (5-FU)-based chemotherapy for high-risk stage II and stage III CRCs. For metastatic stage IV disease, surgical removal of the primary CRC and/or metastatic lesions is followed by therapy using a variety of chemotherapy and targeted treatments. Thus, the mortality rates for each stage, 8–13% (stage I/II), 11–47% (stage III) and approximately 89% (IV), represent the limitations of initial diagnoses and current treatments, indicating that more precise diagnostic measures and effective treatments are required.

Recently, remarkable progress has been made in two key areas at the immunology-cancer interface and microenvironment. This may have a high impact on future diagnoses and treatments for CRC.

First, during the last decade, the relationship between the patient's prognosis and the immunological landscape in primary CRC determined by high-throughput quantitative measurements of cellular and molecular characteristics has been examined through various studies [3–9]. The results of these studies indicate that the inflammatory/immunological response in CRC is heterogeneous among patients [4,5]; an enhanced T-lymphocytic reaction in tumor tissues, especially in the generation of mature memory T cells, reflects an improved prognosis [6,7,10]; but cancer-associated fibroblasts (CAF) in tumor tissues antagonize T-cell antitumor activity and negatively contribute to patients' prognoses [9]. The balance between these two factors may determine disease outcome to a large extent; a classification of CRCs according to their immunological status of tumor microenvironment may accurately predict patient outcome and identify patients with stage I/II/III CRCs for whom there is a high and low risk of recurrence after surgery.

Second, immunotherapies aimed at stage IV cancers using immune checkpoint inhibitors including anti-CTLA-4 antibody, anti-PD-1 antibody and anti-PD-L1 antibody have been revolutionizing cancer treatment [11–13]. In CRC, it has been shown that a microsatellite instable (MSI) subset but not a microsatellite stable (MSS) subset of cancer responds well to checkpoint inhibitor immunotherapy [14]. Thus, the effects of these immune checkpoint inhibitors strongly suggest that the adaptive immune response, even in stage IV disease, plays a critical role for tumor elimination, although its efficacy seems to depend

KEYWORDS

• colorectal cancer • colorectal cancer molecular subtype • DNA mismatch repair • EMAST • immune checkpoint inhibitor • immunotherapy • immunotyping • microsatellite instability • patient outcome • tumor microenvironment

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on the genetic/epigenetic and immunological background of the individual tumor.

Considering that cancer immunotherapy will be a major treatment for cancer patients in the future, understanding of causes and outcomes of heterogeneity in immune response toward cancer is important. One idea is that such immunological heterogeneity in cancer tissues could stem from genetic, epigenetic, transcriptional and translational differences among individual malignant cells. In this review, we sought possible links among host factors including inflammatory/immunological response in tumor tissues, molecular subtypes and patients' prognoses and treatment responses. Toward this goal, literature relating to inflammatory/immunological responses in CRC, molecular subtyping of CRC and current and future immunotherapies for CRC has been compiled.

The CRC–immune microenvironment

Solid tumor tissues consist of different types of cells including malignant cells, innate immune cells (granulocytes, mast cells [MCs] and monocytes/macrophages), adaptive immune cells (T and B cells), fibroblasts and endothelial cells. These cells, either by themselves or together with other cell types, contribute to the inflammatory and/or immunological status of tumor tissues through cell-to-cell contact and/or cytokine/chemokine production. Tumor infiltrating lymphocytes (TIL) are mixtures of T cells, B cells, natural killer (NK) cells, macrophages and other innate cells in variable proportions, T cells being the most abundant. In the early 1900s, the idea that TIL represents a host defense mechanism and that its abundance in tumor tissues is related to cancer patients' survival was formed [15]. By the end of 1970s, evidence supporting this hypothesis was accumulated for many cancer types including CRC [16–18]. In the late 1970s and early 1980s when subpopulations of lymphocytes and their functions began to be identified through cell surface antigen, CD [19], two important observations were made. First, TILs isolated and amplified *in vitro* from some but not all tumor tissues contain T cells expressing $CD4^+$ (a marker for helper T cells) or $CD3^+$ (a marker for total T cells), that specifically recognize and eliminate autologous tumor cells [20–22]. Second, it was recognized that the factors and/or cells that negatively regulate cytotoxic effects of TIL exist in the tumor microenvironment [23,24].

Immune findings associated with improved CRC prognosis

In line with early observations, Jass showed for the first time that a high level of TILs is an independent factor for the survival of rectal cancer patients **(Table 1)** [25]. Ogino *et al.* later demonstrated that higher levels of lymphocytic reactions including Crohn's-like lymphoid reactions, peritumoral reactions, intratumoral periglandular reaction and TILs were associated with patients' prognoses [26]. These observations confirmed the idea that the presence of a high level of lymphoid reaction in CRC tissues is associated with an improved prognosis. Although T lymphocytes were believed to be a key component of TILs, the exact identity of the subpopulation of lymphocytes important for patients' survival was not determined through these studies.

In general, the transformation of naive $CD4^+$ T cells into Th1 cells is triggered by specific binding of their T-cell receptors (TCR) to tumor-derived antigens with class II MHC presented by antigen-presenting dendritic cells (DC). IL-2 and IFN-γ produced by antigen-activated Th1 T cells promote priming and expansion of $CD8^+$ effector T cells. Naive $CD8^+$ T cells are primed to effector T cells expressing high levels of perforin and granzymes when their TCR

Table 1. Factors associated with improved and poor immunological tumor microenvironment.

Improved survival	Poor survival
High level of lymphocytes	Low level of lymphocytes
High level of $CD8^+$ T lymphocytes	Low levels of $CD8^+$ and $CD45RO^+$ T lymphocytes
High level and density of $CD45RO^+$ $CD8^+$ T lymphocytes	High levels of myeloid-derived suppressor cells
Th1 helper T/effector $CD8^+$ T-cell expression	High levels of mast cells
Granzyme B expression	High levels of Th17 cells as tumor infiltrating lymphocytes
High levels of T-cell homing factors and adhesion molecules	CAFs that produce immunosuppressive factors

CAF: Cancer-associated fibroblast.

Table 2. CRC subtyping, immunological landscape, and prognosis of primary CRCs.

TCGA	ClassicalMSI[†]	Immunological characteristics[‡]	Prognosis[§]	CMS[¶]	MSI-H/MSI-L/EMAST[#]
Hypermutated					
MMR-defect	MSI-H-1	T^{++}, HLA^{+}, CAF^{+}	Good	CMS1	MSI-H
12%	MSI-H-2	T^{-}, HLA^{-}	Bad	MSI-H subsets of CMS3 and CMS4	
POLE mutation					
3%	MSS^-1	T^{+}, CAF^{-}	?	?	
	MSS^-2	T^{-}, HLA^{-}	?	?	
Nonhypermutated					
85%	MSS-1	T^{+},CAF^{++}	Bad	CMS4	MSI-L/EMAST without 9p24.2 LOH
	MSS-2	T^{++},CAF^{+}	Good	MSS subset of CMS1	MSI-L/EMAST/9p24.2 LOH
	MSS-3	T^{-}, HLA^{-}, CAF^{-}	Bad	CMS2 and/or MSS subset of CMS3 (KRAS mutation)	Non-MSI-L/EMAST

[†]There are at least two sets of MSI-H CRCs whose immunological landscapes and prognosis are different (MSI-H-1 and MSI-H-2). Likewise, there are two sets of MSS^ CRC whose immunological landscapes are different (MSS^-1 and MSS^-2).

[‡]T^{++} represents the highest infiltration of cytotoxic T cells. T^{+} represents significant level of cytotoxic T-cell infiltration. T^{-} represents no significant level of cytotoxic T-cell infiltration. CAF^{++} represents the highest expressions of CAF markers. CAF^{+} represents significant expressions of CAF markers. CAF^{-} represents no significant levels of CAF marker expressions.

[§]?: Prognosis of MSS^ CRCs is not determined.

[¶]CMS1, CMS2, CMS3 and CMS4 CRCs contain 74, 2, 13 and 11% of total MSI-H CRC (380 cases), respectively, and 24% of CMS1 is MSS CRC in Guinney's study [63].

[#]Hypothetical assignment of MSI-L/EMAST CRCs to MSS-1, -2, or -3 and CMS-1, -2, -3 or -4 CRCs.

CMS: Consensus molecular subtype; CRC: Colorectal cancer; EMAST: Elevated microsatellite alterations at selected tetranucleotide repeat; LOH: Loss of heterozygosity; MMR: Mismatch repair; MSI-H: Microsatellite instability-high; MSS: Microsatellite stable; TCGA: The Cancer Genome Atlas.

specifically binds to the antigen presented with class I MHC on the DC [27,28].

Naito *et al.* first described the infiltration of cytotoxic $CD8^{+}$ T cells within CRC cell nests as an independent prognostic factor [29]. Later, Pages *et al.* showed that CRC tumors without signs of early metastasis (vascular-lymphatic invasion and perineural invasion) exhibit higher densities of Th1 helper T cells, early memory and effector-memory $CD45RO^{+}$ $CD8^{+}$ T cells compared with the CRCs with vascular-lymphatic invasion and perineural invasion. High levels of $CD45RO^{+}$ cells in the tumor microenvironment correlated with increased disease-free survival (DFS) and overall survival (OS) of CRC [3]. Galon *et al.* later reported that tumor recurrence in CRC inversely correlates with high expressions of genes including *TBX21*, *IRF1*, *IFNG*, *CD3-ζ*, *CD8A*, *GZMB* and *GLNY* that are components of Th1 helper T/effector $CD8^{+}$ T-cell functions; a high density of T lymphocyte subpopulations including CD45RO (memory), GZMB (cytotoxic effector), CD8 (effector) and CD3 (total T cells) in either the center of the tumor (CT) or invasive margin of the tumor (IM) is associated with prolonged OS; and a combined analysis of tumor region (CT and IM) for the density of each of T-cell subpopulation had superior prognostic value compared with using classical histopathological parameters (Union for International Cancer Control [UICC]-tumor node metastasis classification) [4]. Galon's group also showed that high-risk stage I/II CRCs are identifiable as CRCs containing low levels of $CD8^{+}$ and $CD45RO^{+}$ T lymphocytes at CT and IM sites [6]. Furthermore, Mlecnik *et al.* showed that a low 'immunoscore' determined by density of $CD3^{+}$ and $CD8^{+}$ at CT and IM sites of primary CRC is associated with the metastatic potential of tumors [8]. They also showed that the high levels of gene expression of T-cell homing factors including chemo-attractants (*CX3CL1*, *CXCL9* and *CXCL10*) and adhesion molecules (*ICAM1* and *MADCAM1*) are associated with a high density of antitumor T cells in tumor tissues, and DFS [30]. All together, these retrospective studies suggest that the activation of Th1 helper and cytotoxic memory T cells plays a key role in preventing recurrence and/or metastasis in CRC **(Table 1)**.

Immune findings associated with poor CRC prognosis

Although improved prognosis of CRC seems to be associated with a high level of antitumor T-cell activity, a large number of CRCs exhibited a decreased level of antitumor activity and associate with a shorter patient survival time. For instance, approximately 60% of stage I/II/III CRC showed a low level of antitumor

immune reaction and approximately 80% of stage IV CRC exhibited a high mortality rate in a large cohort studied by Rozek *et al.* [31]. Furthermore, whereas the level of antitumor T-cell activity decreases with stage, the density of B cells and of innate immune cells, such as neutrophils, MCs, macrophages and immature DCs, increases [32,33]. Angelova *et al.* showed that the presence of myeloid-derived suppressor cells (MDSCs), MCs and Th17 in TILs are significantly associated with stage progression and poor prognosis of CRC **(Table 1)** [34]. MDSCs are immature myeloid cells (macrophage, DCs or neutrophils), generated in the bone marrow (polymorphonuclear-MDSC) and/or spleen (monocytic-MDSC) in response to tumor-derived factors including cytokines, chemokine and metabolites [35]. Migrated MDSCs in various tissues including tumors are suppressive to T-cell antitumor immunity through the increased production of arginine, reactive oxygen species and nitric oxide and the induction of Treg cells and TGF-β secretion [36]. MCs influence tumor angiogenesis, tumor invasion and immune suppression, and contribute to an immune suppressive tumor microenvironment. MCs in cancer promote angiogenesis and tumor invasion into surrounding tissues [37]. Thus, CRC patients with high microvessel and MC densities had significantly poorer prognoses than patients with low microvessel and MC densities [38]. Th17 cells are differentiated from naive $CD4^+$ T cells upon IL6 and TGF-β stimulation. IL-6 induces IL-21, which promotes the expression of the transcription factor RORγt together with IL-23, resulting in IL-17 production. CRC patients with high expressions of Th17 cell genes such as *IL17* and *RORC* exhibited poor prognosis **(Table 1)** [33,34]. However, Amicarella *et al.* reported that the presence of IL-17-producing cells in the intra-epithelial region is associated with an improved prognosis [39]. Thus, further

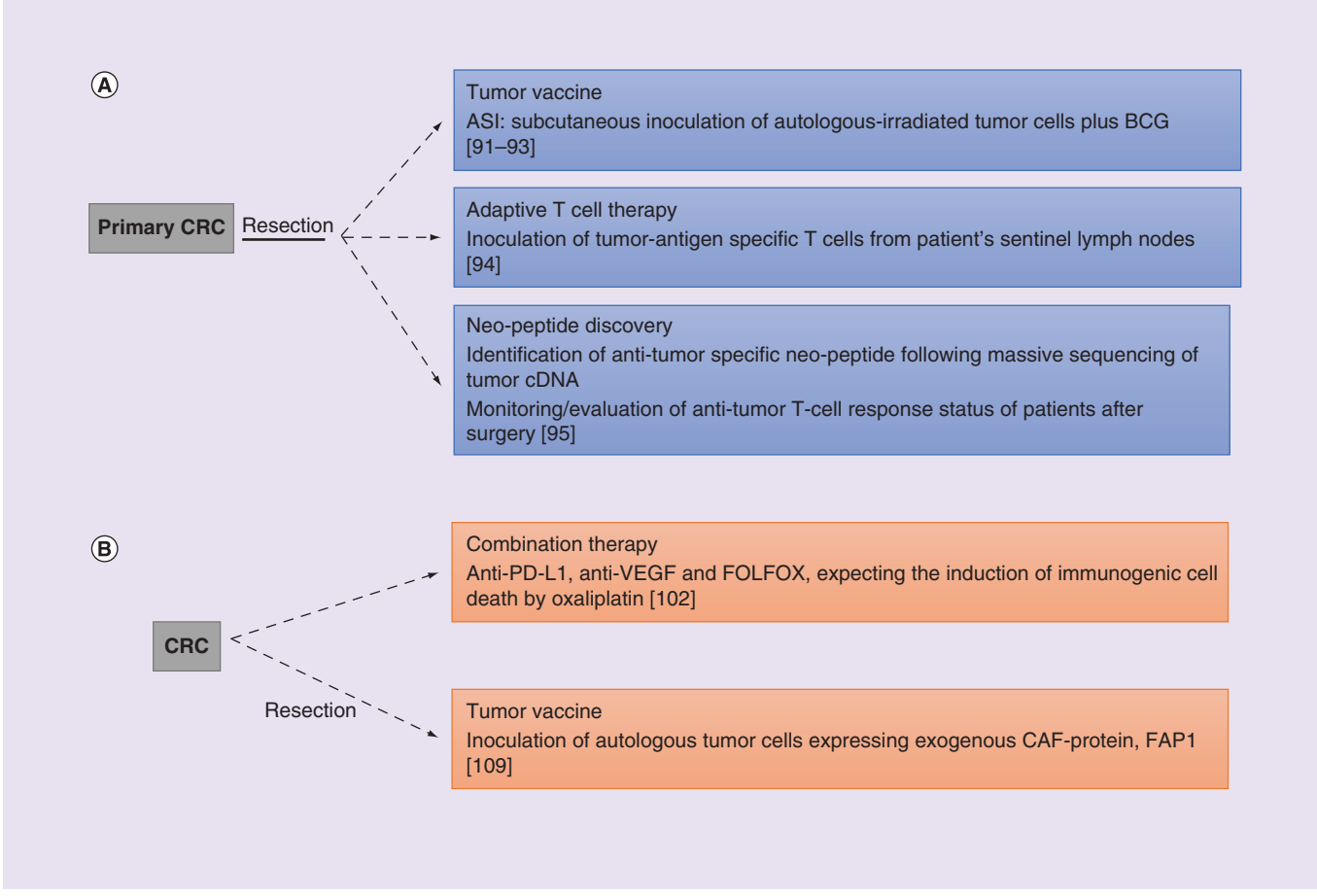

Figure 1. New Immune-based therapies for primary and metastatic CRC. (A) New adjuvant therapies for preventing/monitoring recurrence followed by resection of primary CRC. **(B)** New therapies for treating metastatic CRC.

study is necessary to determine the role of Th17 cells in immune-suppression of CRC. Treg cells expressing *FOXP3* are derived from naive $CD4^+$ T cells upon TGF-β and IL-2 stimulation. Treg cells in TILs from CRC not only produce TGF-β and IL-10 with immune-suppressive activity but also express several immune checkpoints including PD-L1, PD-L2 and CTLA-4 to inhibit effector T-cell functions [40]. Expression of genes characteristic to TIL-Treg cells in CRC is negatively associated with patient survival [40].

The molecular signature of cancer-associated fibroblasts (CAFs) is over-represented for a subset of CRC, which shows the worst prognosis **(Table 1)** [41]. CAFs were found to produce immunosuppressive factors such as LGALS, CXCL12 and PTGS1; proangiogenic factors such as VEGFB, VEGFC and PDGFC; and inflammatory factors such as CCL11, CCL8, CCL2, SAA3 and CXCL5 [41,42]. Taken together, the above studies suggest that an inflammatory environment with depressed or loss of antitumor reactions is associated with a poor prognosis of CRC.

Immune microenvironment associated with specific CRC molecular classifications

Genotypic analysis has identified several CRC subtypes that show specific characteristics in histology, treatment effects and prognosis and survival. Each of these also manifest variations in the immune microenvironment. Broadly, about 15% of all CRCs can be described as hypermutated (containing hundred to a thousand somatic mutations) and about 85% of all CRCs are nonhypermutated (generally containing 60 or less somatic mutations [43,44]. Within hypermutated CRCs are microsatellite instability-high (MSI-H) tumors that are caused by somatic hypermethylation of the DNA mismatch repair (MMR) gene *MLH1*, itself leading to nonfunctional MMR with subsequent accumulation of multiple somatic mutations. The MSI-H is detected biochemically at mono- and di-nucleotide microsatellite frameshifts, as MMR is responsible for correcting point mutations and frameshift slippages at microsatellite sequences after DNA is synthesized during the S phase of the cell cycle [43–47]. The hereditary condition known as Lynch syndrome showcases germline mutations in MMR genes (e.g., *MLH1*, *MSH2*, *MSH6* and *PMS2*) and Lynch-like patients demonstrate double somatic MMR gene mutations that both cause MSI-H and are hypermutated [48]. MSI-H CRCs generate immunogenic neopeptide antigens from the approximately 300 frameshifted, mostly mononucleotide coding microsatellites within genes, with several of the affected proteins abrogating signaling pathways that subsequently drive the progression of these tumors [43,49,50]. Mutations in *POLE* (encoding DNA polymerase e) also cause a hypermutated CRC, but without MSI-H there is no associated defect in MMR [42]. Nonhypermutated CRCs lack MMR gene and *POLE* mutations, and often demonstrate somatic inactivation of *APC*, *TP53* and *SMAD 2/4*, and somatic activation of *KRAS* and *PIK3CA* that drive the progression of these CRCs[43]. Nonhypermutated CRCs, such as CRCs with *POLE* mutations, are MSS, and can be further classified by microenvironment immunologic influences on the subcellular location of the MMR protein MSH3[45,51,52]. Approximately 50% of MSS CRCs show loss of nuclear MSH3 expression, shifted to the cytosol by oxidative stress and the pro-inflammatory cytokine IL-6, which allows the affected cells to accumulate di-, tri- and tetranucleotide frameshift mutations as a result of loss of function of MSH3 [51,53]. The MSH3 defect encompasses the biomarker elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) and MSI-low (MSI-L), a manifestation of one dinucleotide marker frameshifted when using a panel of mono- and dinucleotide markers to assess for MSI-H CRCs[45,54–58]. Importantly, EMAST CRCs are significantly infiltrated with $CD8^+$ T cells compared with non-EMAST CRC [59]. This suggests that tumor microenvironment of MSI-L/EMAST CRCs may be immunologically active. EMAST CRCs demonstrate poor survival [60,61] but can be further modified by loss of heterozygosity (LOH) at chromosome 9p24.2, which improves survival over patients who retain 9p24.2 [62].

Gene expression-based subtyping of CRC has not been standardized among different studies. To obtain unbiased subtyping of CRC, six independent datasets for CRC gene expression were put together and four consensus subtypes with distinguishing features were generated [63]. These include consensus molecular subtype 1 (CMS1), characterized by MSI-H and high T-cell immune reactions (MSI-like: 14%); CMS2, characterized by WNT- and MYC-signaling activation (canonical: 32%); CMS3, characterized by epithelial-gene expression and metabolic dysregulation and enriched by *KRAS* mutations (metabolic: 13%); and CMS4, characterized by significant expression of TGF-β,

stromal-specific expression and angiogenesis (mesenchymal: 23%). Thirteen percent of CRCs showed mixed features [63]. When compared with The Cancer Genome Atlas (TCGA) subtype and MSI subtype, CMS1 corresponds to hypermutated CRC (MSI-H and MSS^), and CMS2, CMS3 and CMS4 correspond to nonhypermutated CRC (MSI-L/EMAST and non-MSI-L/EMAST) (Table 2). Thus, nonhypermutated CRC consists of CMS2, CMS3 and CMS4 and is a transcriptionally heterogeneous population. Among these subtypes, CMS4 exhibits the worst prognosis for DFS and OS. On the other hand, CMS1 (MSI-H) exhibits the shortest survival rates while CMS2 exhibits the longest survival rates after relapse [63].

MSI-H, hypermutated & CMS1 CRCs

As shown in Table 2, MMR-deficient CRCs are MSI-H, hypermutated and enriched in the CMS1 subtype. Through meta-analysis of 32 studies that examined 7642 cases including 1277 MSI-H tumors [64], Popat *et al.* showed that MSI-H CRC exhibits better OS than MSI-L/MSS CRC does. However, recent integrative analysis by Mlecnik *et al.* indicated that MSI-H CRC is heterogeneous and consists of at least two subgroups; a major group exhibiting improved prognosis with high T-cell activity (corresponds to CMS1: MSI-H-1) and a minor group exhibiting poor prognosis with reduced T-cell activity (corresponds to MSI-H in CMS3 and CMS4: MSI-H) (Table 2)[65]. Using sets of gene clusters that identify each of 28 immune cell subpopulations (immunome), Angelova *et al.* analyzed TCGA CRC data to identify a dominant immune cell subpopulation associated with a particular molecular subtype of CRCs. They found that TILs from MSI-H CRC tumors are enriched by lymphocytes with antitumor functions including central and effector memory $CD4^+$/$CD8^+$ cells, gamma delta T cells, Th1, Th2, Treg, follicular helper T cells, immature and memory B cells and NK T cells, compared with TILs from MSS CRC. They also showed that hypermutated MSS CRC with *POLE* mutations (MSS^) was characterized by lower levels of effector memory and central memory $CD4^+$ and $CD8^+$ cells compared with MSI-H CRC, suggesting that the prognosis between these two types of CRC may be different [34]. Becht *et al.* determined the immune landscape of each CMS [9]. As expected, they found that MSI-H-rich CMS1 contains highly immunogenic tumors that elicited an antitumor adaptive immune response. CMS1 was rich in $CD8^+$, B cells and macrophages but low in myeloid and endothelial cell signatures and in the expression of angiogenesis-inducing genes (Table 2). CMS1 also showed the highest PD-1 and HLA class I gene expressions [9].

Since HLA expression is required to activate tumor antigen-specific cytotoxic T cells, abnormalities in the HLA complex (HLA class I and beta 2-microglobulin [B2M]) and in antigen-processing machinery (APM) represent a mechanism by which tumors could escape from antitumor immune surveillance. CRCs with high levels of antitumor T-cell activity and a heavy load of neo-antigens may face stronger selective pressure to evade this activity. Thus, alterations in the HLA complex and APM may occur more frequently in CRCs including MSI-H and MSS^, and may contribute to the progression of tumors. Mutations in *B2M* in the MSI-H CRC cell line and tissues were first reported by Bicknell *et al.* [66]. Later, *B2M* mutations were found to be associated with MSI-H CRC [67]. In addition to *B2M* mutations, loss or down-regulation of HLA class I loci was also found in approximately 60% of MSI-H CRC [68]. Recently, Giannakis *et al.* showed that mutations in the HLA allele were found in 10% (66/~600 cases) of total CRC, and approximately 80% (52/66) of mutations were detected in hypermutated CRCs [69]. Most of the mutations affected the peptide binding and TCR binding domains of HLA, suggesting that abrogation of T-cell interaction is a main consequence of these mutations [69]. They also found mutations in genes involved in APM machinery such as *B2M*, *HSPA5*, *PDIA3*, *TAP1* and *TAP2* [69]. Thus, this immune evasion mechanism may partly explains the immunological heterogeneity seen in MSI-H CRC and in some MSS CRC (Table 2). Recently, increasing evidence has been accumulated to support the view that microbiota in the colon and rectum play important roles not only in shaping the inflammatory microenvironment and promoting CRC development, but they may also modify the efficacy of various therapies [70,71]. Specific bacterial organisms such as *Fusobacterium nucleatum*, *Bacteroides fragilis* and colibactin-producing *Escherichia coli.* are associated with CRC [70,72]. A heavy load of *F. nucleatum* is associated with MSI-H CRC[72–74], and thus is also associated with the proximal colon [74,75]. However, in contrast to most of the MSI-H CRCs, MSI-H CRCs with

high *F. nucleatum* loads exhibit a tumor-microenvironment with a low density of $CD3^+$ T cells [76], and patients with this type of CRC have shorter survival rates [74]. Mechanistically, *F. nucleatum* may induce cell death in human lymphocytes[77] and/or may generate proinflammatory microenvironments [78,79]. Considering that an MSI-H tumor that has lost HLA-1 may not attract T cells in its microenvironment but instead becomes a target of NK cells [80], it may be that *F. nucleatum* protects such tumors from NK cell attack through the binding of its Fap2 protein to the inhibitory receptor, TIGIT (T-cell immunoglobulin and ITIM domain), thus influencing patients' prognoses [79]. This can be easily tested. Further studies are necessary in order to understand the specific association between *F. nucleatum* and MSI-H CRC.

Prognosis & immunological landscape of heterogeneous MSS CRCs

As outlined above, MSS CRC is a genetically (MSI status and mutation loads) and transcriptionally (CMS1, CMS2, CMS3 and CMS4) heterogeneous population. Several studies demonstrated that the immunological landscape of MSS CRC is also heterogeneous, and a subset of MSS CRC exhibits TILs with high memory and cytotoxic T cells [31,41,65,67]. Mlecnik *et al.* showed that MSS CRCs with high densities of $CD8^+$ and $CD45RO^+$ T cells exhibit improved DFS and OS [65]. Rozek *et al.* also observed that the presence of MSS CRC with high density TIL improved OS [31]. On the other hand, Becht *et al.* demonstrated that the presence of MSS CRC with high Th1/$CD8^+$ T cells exhibited poor prognoses that were enriched in CMS4 [9]. They also showed that CMS3 that is rich in *KRAS* mutants, and contains a sub-population of MSI-H CRC, has fewer immunogenic tumor cells with low expression of HLA class I gene, and CMS2 contains fewer immunogenic tumor cells [67]. These observations suggest that there are at least three subsets of MSS CRC, a subset having high Th1/$CD8^+$ T cells with the worst prognosis (enriched in CMS4: MSS-1), a subset with a good prognosis (MSS which could be included in CMS1: MSS-2) and a subset having low Th1/$CD8^+$ T cells with a bad prognosis (may be enriched in CMS2 and/or CMS3: MSS-3) **(Table 1)**. Importantly, subtyping CRC solely based on the density of T-cell activity (immunoscore or TIL density) may miscategorize CMS4 CRC as a CRC with a good prognosis [31,41,65].

Becht *et al.* further showed that CMS4 CRCs with the worst prognosis had a high expression of antitumor immune signature and were also rich in endothelial and fibroblast cell signatures and in the expression of angiogenesis-inducing genes. CMS4 contains an abundance of cancer-associated fibroblasts (CAF), endothelial cells and monocytes, which produce factors that foster inflammation, angiogenesis and immunosuppression [67]. One of the factors that contributes to a negative impact on survival of CMS4 is TGF-β mediated prometastatic tumor microenvironment[80]. Calon *et al.* showed that stromal-specific gene products such as CALD1, POSTN, FAP and IGFBP7 in CRC can be predictive factors for poor prognoses. They demonstrated that these products were expressed by stroma but not by epithelial tumor cells. Mechanistically, it was suggested that TGF-β produced by tumor cells and CAF itself activates CAF in the tumor stroma to assist tumor cells by increasing their ability to initiate metastasis [81]. Recently, like CMS4 CRC, a subset of clear-cell renal cell carcinoma (RCC) with high $CD8^+$ T cells and a high inflammatory and immunosuppressive microenvironment was shown to exhibit poor prognoses [82]. Of note, most clear-cell RCC tumor cells contain inactivation of the von Hippel–Lindau tumor suppressor function [83], leading to activation of the Hypoxia Inducible Factor (HIF) pathway and production of pro-inflammatory cytokines such as VEGF, IL6 and TGF-β [82]. It is tempting to speculate that CMS4 CRC tumor cells may have a deregulated HIF pathway and produce cytokines that activate CAF, attract polyclonal $CD8^+$ T cells and hamper tumor-specific T-cell activity.

As described earlier, MSS CRCs can be divided into MSI-L/EMAST and non-MSI-L/EMAST CRC [60,62]. Immunologically, MSI-L/EMAST CRCs have been heavily infiltrated with $CD8^+$ but not $CD4^+$ T cells as compared with non-MSI-L/EMAST CRC [59] and are frequently seen in ulcerated CRC [58,59,61], suggesting that the tumor microenvironment of MSI-L/EMAST CRC is inflammatory and immunologically active. MSI-L/EMAST CRC at stage II/III showed poor DFS[60,62]. Furthermore, it is likely that the mechanism inducing MSI-L/EMAST in CRC tumor cells is loss of MSH3 in the replicating nucleus due to exposure to inflammatory

and/or hypoxic tumor microenvironment [51,52,56,58]. Recently, it has been reported that a frequent allelic loss at the *HIF3A* locus whose product negatively regulates HIF1A and HIF2A functions [84] is observed in liver metastasis from MSI-L/EMAST primary CRC, suggesting that deregulation of the HIF pathway may contribute to metastasis from primary CRC [84,85].

However, MSI-L/EMAST CRC is not a homogenous population. Stage III CRC exhibiting MSI-L/EMAST plus LOH at chromosome 9p24.2 showed an improved prognosis compared with the rest of stage III, non-MSI-H CRC [62]. This set of CRCs may overlap with those with high Th1/CD8$^+$ T cells and with improved prognoses detected by Mlecnik *et al.* [65] and Rozek *et al.* [31], and may be a subgroup of CMS1 **(Table 2)**. On the other hand, stage II/III MSI-L/EMAST CRCs without 9p24.2 LOH exhibited poor prognoses [62]. Thus, because characteristics of MSI-L/EMAST without 9p24.2 LOH CRCs are like those of CMS4 CRC, it is tempting to speculate that these subsets of CRC may overlap **(Table 2)**. Non-MSI-L/EMAST CRCs may be enriched in CMS2 and/or CMS3 **(Table 2)**. Further studies are required to test this hypothesis, and to identify the gene affected by 9p24.2 and its function.

Immune-based predictive markers & treatment for CRC

• Developing predictive markers for recurrence of stage I/II/III CRC

Classifying CRCs based on the immunological, genetic and transcriptional landscapes identified through the studies described so far may contribute to lowering the incidence of recurrence and associated death. As mentioned above, on average, approximately 10% of stage I/II and approximately 30% of stage III CRC patients experience recurrence with distant metastasis after curative surgery. To reduce the recurrence rate, progress could be made in two critical areas. First, a predictive marker(s) that identifies patients at high risk for recurrence should be discovered and developed. Second, new adjuvant therapies, alone or in combination with current 5-FU-based chemotherapy, that are specifically effective to high-risk patients identified by new markers should be developed.

As a predictive marker, the immunoscore system developed by Galon's group seems promising [10]. They showed that high-risk stage I/II CRCs, which gave rise to recurrence after surgery, exhibited a low immunoscore (low levels of CD8$^+$ and CD45RO$^+$ T lymphocytes densities at CT and IM sites) [6]. However, immunoscores alone may not accurately predict patient outcomes; immunoscores cannot distinguish between CRCs with high levels of Th1/CD8$^+$ T cells that exhibit poor prognoses (CMS4 subtype CRC) and those that exhibit improved prognoses. On the other hand, expression of CAF markers including CALD1, POSTN, FAP and IGFBP7 can be a predictive factor for poor prognosis in CRC [81]. Another potential marker would be MSI-L/EMAST that is associated with shorter DFS of stage II non-MSI-H CRC [62]. Thus, it would be feasible to develop a new marker system that combines immunoscore, CAF markers and MSI-L/EMAST markers to more accurately identify high-risk stage I–III CRC.

Immune-based adjuvant therapies for stage I/II/III CRC

There is an urgent need to develop new adjuvant treatments that will eradicate tumor cells already disseminating from a primary site before surgery, potentially escaping from 5-FU-based adjuvant therapy and forming micrometastasis in other tissues. One promising and a cost-effective treatment, if proved, is the use of aspirin and/or NSAIDs as adjuvant therapy for CRC [86,87]. Many observational studies support the idea that a daily aspirin use elongates a cancer-specific survival after diagnosis of CRC [87]. The study by Hamada showed that the effect of aspirin use is associated with the expression levels of PD-L1 [88]. Another study by Gray *et al.* showed that efficacy of aspirin is associated with levels of PTGS2 expression in CRC [89]. These studies suggest that a response to aspirin is heterogeneous in CRC population. Currently, five trials (ISRCTN74358684, NCT02647099, NCT00565708, NCT02301286 and NCT02467582) are ongoing worldwide to determine the adjuvant effect of aspirin on CRC [87]. Using a mouse model of prostate cancer metastasis, Kwon *et al.* demonstrated that administration of anti-CTLA-4 antibody following complete resection of primary tumors significantly inhibited recurrence of residual metastatic growth, suggesting that immune-based therapies can be used for controlling recurrence after surgery [90]. There are several trials and pilot studies testing an immunological approach for preventing recurrence of CRC. Adjuvant active specific immunotherapy with an autologous tumor cell – Bacillus Calmette-Guérin vaccine – was conducted to determine whether surgical resection plus active specific immunotherapy was more

beneficial than resection alone in stage II and III colon cancer patients **(Figure 1A)** [91–93]. The results of these studies showed that co-inoculation of irradiated, viable tumor cells from stage II colon cancer exhibiting MSS but not MSI-H, and viable Bacillus Calmette-Guérin significantly prolonged DFS. Based on these studies, a randomized, multicenter Phase III study is underway (NCT02448173). Zhen *et al.* have started Phase I/II study examining the prognostic effect of reinfusion of TILs isolated from sentinel lymph nodes in patients with stage I-IV CRC after surgery followed by 5-FU adjuvant chemotherapy. They found that these procedures are feasible and safe, and obtained promising results showing survival benefits of these procedures for stage IV CRC **(Figure 1A)** [94]. Another encouraging approach was reported by Mennonna *et al.* in which they first identified mutated expressed genes in tumors from each patient with MMR-proficient CRC. Then, they co-cultured patients' peripheral blood cells with a pool of a long overlapping peptides encompassing somatic mutations found in the patient's tumor, or with autologous tumors expressing the mutations, and detected mutation-specific $CD8^+$ and $CD4^+$ T cells in one patient with an improved prognosis but not in another with poor prognosis [95]. These results suggested that memory T cells that recognize autologous tumors exist in the peripheral blood of some but not all patients with MSS CRC. Although further work using more patients and more genes to be sequenced are necessary, this approach could be used to characterize antitumor immune responses in patients undergoing surgery, and also to define neo-antigens for effective tumor vaccines or to obtain tumor-specific effector T cells for adaptive cellular therapy **(Figure 1A)** [95]. Considering heterogeneity in immunological landscape among individual CRCs, effective treatments using an immune-based approach would vary for each case. Although further studies are needed to find effective treatments for each case, it is important to note that resected tumor tissues including nearby lymph nodes are a valuable source not only for determining prognosis of patients but also for obtaining materials to raise tumor vaccines, adaptive cellular therapy specific to the patient.

Immune-based therapy to enhance antitumor activity for stage IV CRC

At the time of diagnosis, more than 20% of patients have metastatic CRC (mCRC) (synchronous metastasis) and 18% of patients originally diagnosed as stage I/II or III CRC have recurrent metastasis (metachronous metastasis) within 2–3 years after surgery. The mortality rate exhibited by mCRC (stage IV) is approximately 88% under current practice. Thus, new therapies that not only elongate patients' survival but also potentially eradicate metastatic tumors are needed.

Toward this goal, many therapies based on small-molecule inhibitors or monoclonal antibodies are under clinical development [96]. 'Targeted therapies' aim to shut down upregulated signaling pathways that contribute to tumor growth or to activate cell death pathway. These include targeting *BRAF* mutations and various signaling pathways such as MET, IGF, MEK, PI3K, WNT, Notch, Hedgehog and TGF-β and activation of death-receptor 4 and 5 pathways [96]. The second type of therapy aims to enhance antitumor activity. These approaches include enhancing antitumor T-cell function, enhancing antitumor innate cell function and targeting tumor-associated macrophage [96]. Recently, the approach aims to enhance antitumor T-cell function by administration of immune-checkpoints inhibitors have had remarkable success in treating various metastatic cancers including melanoma, non-small-cell lung carcinoma, RCC, bladder carcinoma and Hodgkins lymphoma [12,13,97].

• Immune-checkpoint inhibitors

CTLA-4 is constitutively expressed in Treg cells and upregulated in T cells when they are activated. Its function is to inhibit T cell-mediated immune response. Thus, CTLA-4-positive T or Treg cells contribute to immune tolerance to tumor cells. PD-1 is expressed on T, B, macrophages and some DCs. PD-1 binds to PD-L1 and PD-L2. PD-L1 is expressed in a broad range of hematopoietic and nonhematopoietic cells. After binding to these ligands, PD-1 primary inhibits effector T-cell activity whereas CTLA-4 mainly inhibits early stage of T-cell activation. The inhibitory effect of PD-1 is accomplished through a dual mechanism of promoting programmed cell death of antigen-specific T cells while promoting *de novo* generation of Tregs under the presence of TGF-β [98].

Despite recent success in cancer immunotherapies targeting immune checkpoints such as CTLA-4, PD-1 and PD-L1 in many cancers, early trials examining the clinical efficacy of anti-CTLA-4 antibody treatment and anti-PD-1 antibody treatment on mCRC gave disappointing results; the former gave rise to one case with a

partial response (PR) among 47 cases treated [99] and the latter gave rise to one case with complete response among 33 cases treated [12,100]. Anti-PD-L1 antibody treatment on mCRC gave negative results when 18 patients were treated [13]. These results suggest that monotherapy using immune checkpoint inhibitors alone may not be effective on most of mCRCs.

Le *et al.* found that one of the 33 mCRC cases that showed complete response to PD-1 blockade in early studies was MMR-deficient, MSI-H CRC. They extended this observation and showed that MMR status in mCRC predicts clinical benefits of PD-1 blockade [14]. Their results also showed that somatic mutation loads were associated with clinical efficacy of anti-PD-1 antibody treatment [14]. Recently, Overman *et al.* reported that monotherapy with PD-1 blockade was more effective in MSI-H mCRC group (25.5% PR rate: 12 of 47 cases) compared with non-MSI mCRC group (10% PR rate: 1–10 cases). They also showed that combination therapy with PD-1 and CTLA-4 blockades resulted in 33.3% PR (9 of 27 cases) in MSI-H group and 0% PR (0 of 10 cases) in non-MSI-H mCRC [101].

To date, clinical trial studies performed show a patient benefit with mCRC for CTLA-4, PD-1 or PD-L1 blockades, alone or combination, but is limited to the MSI-H subset. Considering that the MSI-H subset is only a small fraction (4%) of mCRC, and the response rate of blockade-treated MSI-H is between 25 and 35%, only approximately 1% of mCRC is expected to respond to these treatments.

However, a recent Phase I study testing the efficacy of PD-L1 blockade combined with conventional chemotherapy on mCRC gave promising results. Bendell *et al.* reported that 40% (12/30) of patients treated with a combination of anti-PD-L1 antibody, anti-VEGF antibody and FOLFOX exhibited PR while only 8% (1/13) of patients treated with anti-PD-L1 and anti-VEGF antibodies without FOLFOX exhibited PR **(Figure 1B)** [102]. These results highlight the importance of chemotherapy that reinstates immune-surveillance when immune-based therapies are applied for immune-depressed tumors [103]. Of note, oxaliplatin is known to induce immunogenic cancer cell death, which reactivates an antitumor immune response [104]. 5-FU selectively eliminates immunosuppressive MDSC, which accumulates during cancer progression [105]. The next important step would be to identify common tumor characteristics including immunological landscape, molecular subtypes or MSI/EMAST status, if any, shared by the 12 cases of mCRC that responded to the combination therapies. It is tempting to speculate that mCRCs susceptible to combination therapy between immune checkpoint inhibitors and conventional anticancer drugs may be derived from primary CRCs that infiltrated with Th1/CD8$^+$ T cells such as CMS4 subtype CRC [67,81] and/or MSI-L/EMAST with 9p24. LOH that behave less aggressively even after the tumor cells metastasized to the liver [62]. Answers to this question may give new insight for treating stage IV CRC.

• Therapy-targeting CAFs

Molecular subtyping of primary CRC identified the CMS4 subset that exhibited the worst prognosis of all subsets. Although CMS4 primary CRC was significantly infiltrated with Th1/CD8$^+$ T cells, poor prognosis of CMS4 is associated with a preponderant presence of CAFs [81]. Assuming that mCRC may more or less develop a CAF-enriched tumor microenvironment at both primary and metastasized sites, development of therapies targeting CAFs is necessary [106]. FAP expressed by CAF has been a target of several preclinical studies and clinical trials [106–108]. Phase I study using human monoclonal anti-FAP antibody against mCRC with liver metastasis showed no clinical activity in 17 treated patients [109]. However, a recent preclinical study reported by Chen *et al.* showed that inoculation of FAP-transfected whole-cell tumor cells induced strong antitumor immunity against both tumor cells and CAFs in experimental animals. They also observed decreased recruitment of immunosuppressive cells including MDSC, M2 macrophage and Tregs, and enhanced recruitment of effector CD8$^+$ T cells into TME of mice with FAP-transfected tumor cells **(Figure 1B)** [109].

Conclusion

A relationship between the genetic, transcriptional, immunological characteristics and prognosis of primary CRC is present. Th1/CD8$^+$ T cell enriched in the tumor microenvironment found in primary CRC is associated with a reduced incidence of recurrence and/or metastasis while a CAF-enriched tumor microenvironment in primary CRC is associated with a poor prognosis for stage I/II and III CRC. Developing a new classification system of primary CRC based on the current tumor node metastasis system combined with Th1/CD8$^+$ T-cell markers, CAF markers and microsatellite markers would more accurately

and cost-effectively identify high-risk CRCs that might relapse after surgery and could facilitate the discovery of a critical target for adjuvant therapy.

The benefit of immune-checkpoint blockade in treatment of metastatic CRC is currently limited, approximately 1% (mostly MSI-H CRC) of total mCRCs. However, there is an evidence that a subset of mCRCs including MSI-H and MSS CRC may respond to this treatment when combined with conventional chemo and targeted therapies. Conventional chemotherapy or targeted therapy that may reinstate immune-surveillance could be a successful approach when immune-based therapies are applied to immune-depressed tumors like mCRC.

Future perspective

As we gain more insight into the appropriate compendium of biomarkers that will provide accurate and reliable diagnostic and prognostic information regarding the immune influence on CRC, we predict there will be increased and broader uses for immune checkpoint therapy in this disease. The use of immune checkpoint inhibitors will likely move for the most appropriate patients to the adjuvant and first-line metastatic scenarios for treatment. We also predict, as knowledge is gained, that some proportion of nonsensitive CRCs to immune checkpoint inhibitors could be induced to become more sensitive, effectively adding immune checkpoint therapy to the treatment options for these additional subset of patients.

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EXECUTIVE SUMMARY

Background

- Colorectal cancer (CRC) is the third most common cancer worldwide, with mortality rates of 8–13% for stage I–II, 11–47% for stage III and 89% for stage IV, despite current treatments.
- The inflammatory and immunological response observed in CRCs is heterogeneous among patients.
- Immune checkpoint inhibitor use in stage IV cancers has shown that the immune system is important and has a key role for cancers.

The CRC–immune microenvironment

- Patients whose tumors demonstrate tumor infiltrating lymphocytes, particularly Th1 helper and cytotoxic memory T cells, are associated with improved CRC prognosis.
- Patients whose tumors demonstrate Treg and Th17 cells as tumor infiltrating lymphocytes, and/or a molecular signature consistent with cancer-associated fibroblasts, are associated with poor CRC prognosis.
- CRC tumor molecular classification influences the tumor microenvironment.
- Microsatellite instability-high, hypermutated or consensus molecular subtype 1 CRCs tend to be enriched by lymphocytes with antitumor function.
- Microsatellite stable CRCs are an immunological heterogeneous population that can be genetically evaluated into any of the consensus molecular subtype classes to determine prognosis.

Immune-based predictive markers & treatment for CRC

- The immunoscore, a measure of the type and density of the immune response within primary CRC, is an initial promising value as a predictive marker.
- Immune therapies for patients with stage I/II/III CRCs that are being tested include adjuvant active specific immunotherapy and therapy based on specific mutations found in the primary tumor.
- Immune therapies for patients with stage IV CRCs that are being tested include immune checkpoint therapy for sensitive MSI-H tumors and anticancer-associated fibroblast therapy.

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