Review Article

Immunotherapy in Colorectal Cancer: Current and Future Strategies

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Abstract
Despite the recent advances in the systemic treatment of metastatic colorectal cancer (mCRC), prognostic outcomes have remained to be poor. Thus, what is needed is an innovative treatment approach. Immune checkpoint inhibitors (ICIs) targeting programmed death-1 (PD-1) and anti-programmed cell death ligand 1 (PD-L1) have exhibited a durable response and dominated the treatment of various tumor types. However, in mCRC, the clinical benefit is limited in patients with deficient mismatch repair (dMMR)/high levels of microsatellite instability (MSI-H), comprising approximately 5% of mCRC cases, and some do not respond to ICI treatment. Thus, further research is needed to identify predictive biomarkers. The most urgent need is developing effective immunotherapy for patients with proficient mismatch repair (pMMR)/microsatellite stable (MSS) cancer, which comprises 95% of mCRC cases. Tumors with the pMMR/MSS phenotype often exhibit a lower tumor mutation burden and fewer tumor-infiltrating lymphocytes than dMMR/MSI-H, leading to immune tolerance and evasion in the tumor microenvironment. Therefore, a number of investigative studies aimed at overcoming tumor resistance in current immunotherapy approaches are underway. A better understanding on the complexity and diversity of the immune system's functioning within the tumor microenvironment will increase the potential for developing predictive biomarkers and novel therapeutic strategies to potentiate anti-tumor immunity in patients with mCRC. In this review, we summarize the most recent advances in immunotherapy based on the findings of pivotal clinical trials for patients with mCRC, highlighting potent therapeutic approaches and predictive biomarkers.

Keywords
chemotherapy, colorectal cancer, PD-1, PD-L1, CTLA-4, MSI

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1. Introduction

Colorectal cancer (CRC) has been identified as the second leading cause of cancer-related deaths worldwide[1]. Approximately 80%-90% of metastatic CRC (mCRC) patients have been determined to have unresectable disease, and combining chemotherapy and molecular-targeted agents that inhibit vascular endothelial growth factor (VEGF; e.g., bevacizumab) or epidermal growth factor receptor (EGFR; e. g., cetuximab or panitumumab) is the optimal first-line treatment regimen[2]. However, its clinical benefits are limited because mCRC is, essentially, impossible to cure, and the median overall survival (OS) is estimated to be at approximately 30 months[2]. Therefore, further developing novel agents is required to improve prognostic outcomes.

The discovery of inhibitory immune checkpoint molecules that allow tumors to escape from immune surveillance has prompted an innovative revolution in anti-tumor treatment, especially anti-programmed death-1 (PD-1) and anti-programmed cell death ligand 1 (PD-L1)[3]. Immune check-

point inhibitors (ICIs) targeting PD-1/PD-L1 have dramatically changed therapeutic paradigms, as a durable clinical response is achieved by disrupting immune tolerance and activating cytotoxic T-cells in refractory patients with solid tumors, including a small subset of patients with mCRC[4-9]. From the anti-tumor immunogenic perspective, mCRC can be categorized into two types of tumors. The first type is deficient mismatch repair (dMMR) or high level of microsatellite instability (MSI-H), whereas the second type involves proficient mismatch repair (pMMR) or microsatellite stable (MSS)[10]. dMMR/MSI-H CRCs have been determined to exhibit a higher tumor mutation burden and more tumor-infiltrating lymphocytes (TILs) than pMMR/MSS CRCs, making these tumors sensitive to treatment with ICIs[10-12]. Based on the impressive results from clinical trials among patients with dMMR/MS-H[5-9], two anti-PD-1 antibodies, pembrolizumab and nivolumab, have been granted Food and Drug Administration (FDA) approval for patients with dMMR/MSI-H mCRC. However, some patients with dMMR/MSI-H are deemed unsuitable for ICIs, and biomarker selection is, therefore, needed in optimizing the treatment.

Importantly, most patients with pMMR/MSS tumors, which comprise about 95% of mCRC cases, often do not benefit from current immunotherapy approaches. Thus, elucidating the determinant mechanisms of immunotherapy resistance is a must to pave the way for developing new treatment strategies. Currently, several clinical trials are evaluating the efficacy of immunotherapy combined with chemotherapy, radiation therapy, or other agents in enhancing T-cell infiltration into tumors and anti-tumor immunity.

In this review, we have laid down the most recent advances in immunotherapy based on the results of pivotal clinical trials among patients with mCRC and further highlight potent therapeutic approaches and predictive biomarkers.

2. Immune Checkpoint Blockade

2.1. Rationale for targeting immune checkpoint molecules

The immune system plays a key role in eliminating tumor cells. However, the anti-tumor immune response is often determined to be prevented by immune checkpoint molecules, such as anti-cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), PD-1, and PD-L1 during the cancer-immunity cycle process[13]. CTLA-4, which is exclusively expressed on T-cells, acts as a negative regulator of the initial priming of T-cells as it outcompetes CD28 in binding to critical costimulatory molecules (CD80 and CD86), which are located on antigen-presenting cells (APCs)[14]. PD-L1 is, generally, undetectable in normal cells, but inflammatory cytokines, particularly interferon-gamma (IFN-γ), are found to

often stimulate PD-L1 expression on various cell types in the tumor microenvironment. The PD-1 receptor acts as a dominant-negative regulator of anti-tumor T-cell effector functioning, by engaging PD-L1[15].

In the cancer-immunity cycle, once T-cells are activated via specific tumor antigens presented by APCs, they circulate and recognize their cognate antigen that is presented by tumor cells. T-cell receptor (TCR) recognition of cognate antigens presented by major histocompatibility complex (MHC) molecules on the surface of tumor cells induces an anti-tumor response. However, triggering TCRs can lead to PD-1 expression, IFN-γ production, and, subsequently, reactive PD-L1 expression by targeted tumor cells, which turns off anti-tumor T-cell responses, referred to as T-cell exhaustion[14]. Thus, the PD-L1/PD-1 signaling axis induces adaptive immune resistance in the tumor microenvironment, and an immune-based treatment approach using ICIs may, therefore, be beneficial.

2.2. dMMR/MSI-H and pMMR/MSS in CRC

Microsatellites are identified as regions of the genome with multiple short tandem DNA repeats, which are prone to DNA base insertions or deletions due to slippage and errors caused by DNA polymerase during DNA replication, resulting in MSI-H[16]. Since the MMR system plays a key role in recognizing and correcting these errors, dMMR allows the increased accumulation of frameshift somatic mutations. Typically, dMMR/MSI-H CRCs have a 10- to 100-fold greater number of somatic mutations than pMMR/MSS CRCs[12]. Most MSI-H CRCs are sporadic tumors due to the epigenetic silencing of MMR genes. For patients with Lynch syndrome, MSI-H CRC can be inherited due to germline mutations in MMR genes[17]. The prevalence of dMMR/MSI-H is determined to be tumor stage-dependent, as it is higher in Stage II (20%) and Stage III (12%) than in Stage IV (4%) CRC[18]. In Japan, the frequency is found to be slightly lower: 9.0% for Stage II, 4.7% for Stage III, and 2.1% for Stage IV CRC[19]. Over 95% of mCRC patients have been identified to have the pMMR/MSS phenotype.

dMMR/MSI-H CRCs have a distinct pathological profile that includes right-sided primary, mucinous, and poorly differentiated tumors, as well as more BRAF mutations, when compared to pMMR/MSS CRCs[20]. Importantly, dMMR/MSI-H CRCs have the ability to produce a plethora of immunogenic neoantigens on the MHC via the high tumor mutation burden, priming T-cells to recognize them as non-self and recruiting T-cells within the tumor[10]. Furthermore, dMMR/MSI-H CRCs have high TILs with activated CD8+ cytotoxic T-lymphocyte (CTL) and T helper type 1 (Th1) cells characterized by IFN-γ production. Consequently, the activated immune response may contribute to the reduced rates of tumor recurrence and favorable prognostic outcomes in patients with dMMR/MSI-H CRC, compared to those

Table 1. Clinical Outcomes from Pivotal Trials in Patients with dMMR/MSI-H.

	KEYNOTE-177		CheckMate 142			KEYNOTE-164		KEYNOTE-016
Treatment line	1st		1st	≥ 2nd	≥ 2nd	≥ 2nd	≥ 3rd	≥ 3rd
Phase	III		II	II	II	II		II
Number of patients	153	154	45	74	119	63	61	10
Regimen	Pembro	Chemo	Nivo + Ipi*	Nivo	Nivo + Ipi**	Pembro	Pembro	Pembro
ORR	44%	33%	60%	31%	55%	33%	33%	40%
DCR	65%	75%	84%	69%	80%	57%	51%	90%
mPFS (months)	16.5	8.2	NR	14.3	NR	4.1	2.3	NR
12-month PFS rate	55%	37%	77%	50%	71%	41%		78% (at 20 weeks)
mOS (months)	-	-	NR	NR	NR	NR	31.4	NR
12-month OS rate	-	-	83%	73%	85%	76%	72%	-

Abbreviations: dMMR, deficient mismatch repair; MSI-H, microsatellite instability-high; Pembro, pembrolizumab; Nivo, nivolumab; Ipi, ipilimumab; Chemo, chemotherapy; ORR, overall response rate; DCR, disease control rate; PFS, progression-free survival, OS, overall survival; NR, not reached.

* Nivo 3 mg/kg biweekly + Ipi 1 mg/kg every 6 weeks

** Nivo 3 mg/kg + Ipi 1 mg/kg every 3 weeks (four doses) followed by Nivo 3 mg/kg biweekly

with pMMR CRC, in the early stage. However, advanced-stage dMMR/MSI-H CRCs have poor outcomes, deriving less benefit from conventional chemotherapy, partially via immune-resistant mechanisms[21]. dMMR/MSI-H CRCs also stimulate the expression of at least five immune checkpoint molecules, including PD-1, PD-L1, CTLA-4, lymphocyte activation gene 3 (LAG-3), and indolamine 2,3-dioxygenase (IDO), which counterbalance the active function of CTL/Th1 cells and create an immune-evasive state in the tumor microenvironment[10]. These preclinical findings provide a rationale for treating dMMR/MSI-H CRC patients with ICIs.

2.3. Clinical trials of anti-PD-1 antibodies

Results from pivotal trials of patients with dMMR/MSI-H and with pMMR/MSS mCRC have been summarized in Table 1, 2, respectively. Early studies of patients with non-selected mCRC have showed that ICIs have very limited clinical activity. In a phase I study of an anti-PD-1 immunoglobulin G4 (IgG4) antibody, nivolumab, in patients with refractory solid tumors (NCT00729664), no objective response was observed in 18 patients with mCRC[22]. In another phase I study of nivolumab in 39 patients with refractory solid tumors (NCT00441337), only 1 in 14 patients with mCRC had an objective response. Notably, this patient had a complete response that lasted longer than 3 years after retreatment and had mCRC with dMMR/MSI-H[23,24]. In the multicohort phase Ib KEYNOTE-028 trial (NCT02054806), an anti-PD-1 IgG4 antibody, pembrolizumab, was evaluated in patients with PD-L1-positive advanced solid tumors. Only one partial response was recorded among 23 patients with PD-L1-positive mCRC, and the response lasted more than 24 months[25]. Again, this patient has also been identified to have MSI-H mCRC, suggesting that the dMMR/MSI-H status is a predictive marker for ICI response.

Since somatic mutations can encode non-self immunogenic neoantigens, tumors with a high mutational burden due to dMMR may be sensitive to ICIs. Based on this hypothesis, the phase II KEYNOTE-016 trial (NCT01876511) was performed to evaluate the clinical efficacy of pembrolizumab in patients with pMMR/MSS mCRC, dMMR/MSI-H mCRC, or dMMR/MSI-H non-CRC[7]. No response was noted in 18 patients with pMMR/MSS mCRC, whereas the overall response rate (ORR) was 40% in 10 patients with dMMR/MSI-H mCRC. A similar positive effect was observed in the cohort with dMMR/MSI-H non-CRC, with an ORR of 71% (5/7). The updated and expanded results have showed similar trends, in which ORR and the disease control rate (DCR) were 50% and 89%, respectively, for dMMR/MSI-H mCRC (n = 28) and 0% and 16% for pMMR/MSS mCRC (n = 25), respectively. After a median follow-up period of 8.7 months, the median progression-free survival (PFS) and OS were not reached for dMMR/MSI-H mCRC, while the PFS and OS were determined to be 2.4 months and 6.0 months for pMMR/MSS mCRC, respectively[26]. Based on the above proof-of-concept suggesting that a patient's MMR/MSI status can predict the clinical response to ICIs, the efficacy of pembrolizumab was evaluated in 86 patients with dMMR/MSI-H tumors across 12 different types[6]. A complete response (CR) was achieved in 18 of 86 patients (21%), while the ORR and DCR were 53% (46/86) and 77% (66/86), respectively. The estimated rates

Table 2. Clinical Outcomes from Pivotal Trials in Patients with pMMR/MSS.

Trial	Regimen	Phase	No. of pts	MSS rate	ORR		DCR	PFS (months)	OS (months)
KEYNOTE-028	Pembro	II	23	96%	4%		20%	1.8	5.3
CheckMate 142	Nivo + Ipi*	II	10	100%	10%		-	2.3	11.5
	Nivo + Ipi**		10	100%	0%		-	1.3	3.7
CCTG CO.26	Duru + Treme	II	119	98%	1%		23%	1.8	6.6
IMblaze370	Atezo + Cobi	III	183	93%	3%		26%	1.9	8.9
	Atezo		90	92%	2%		21%	1.9	7.1
	Rego		90	100%	2%		34%	2	8.5
BACCI	Cape + Bev + Atezo	II	82	86%	9%	(MSS 8%)	88%	4.4	10.5
	Cape + Bev		46	87%	4%	(MSS 3%)	88%	3.3	10.6
REGONIVO	Nivo + Rego	Ib	25	96%	36%	(MSS 33%)	88%	7.9	68% at 1 year
Kim et al	Nivo + Rego	I	28	100%	5%		71%	4.3	11
REGOMUNE	Avel + Rego	II	48	100%	0%		54%	3.6	10.8

Abbreviations: pMMR, proficient mismatch repair; MSS, microsatellite stable; Pembro, pembrolizumab; Nivo, nivolumab; Ipi, ipilimumab; Rego, regorafenib; Atezo, atezolizumab; Cobi, cobimetinib; Duru, durvalumab; Treme, tremelimumab; Avel, avelumab; Cape, capecitabine; Bev, bevacizumab; ORR, overall response rate; DCR, disease control rate; PFS, progression-free survival, OS, overall survival.
* Nivo 1 mg/kg + Ipi 3 mg/kg every 3 weeks (four doses), followed by Nivo 3 mg/kg biweekly
** Nivo 3 mg/kg + Ipi 1 mg/kg every 3 weeks (four doses), followed by Nivo 3 mg/kg biweekly

at 2 years were 59% and 72% for PFS and OS, respectively. No recurrences were determined at the median follow-up time of 8 months in the 18 patients who discontinued therapy at 2 years after being treated per protocol, suggesting that a PD-1 blockade produces a durable response in patients with dMMR/MSI-H tumors. Importantly, the ORR was found to be similar in mCRC (52%, 21/40) and other non-CRC tumors (54%, 25/46). Tumors with dMMR/MSI-H have been determined to have a much higher number of somatic mutations than those with pMMR/MSS in whole-exome sequencing, and high somatic mutation loads were often associated with treatment efficacy[7]. Furthermore, patients responding to a PD-1 blockade exhibited the clonal expansion of mutation-associated neoantigen-specific T-cells into dMMR/MSI-H tumors[6]. These findings support the hypothesis that patients with dMMR/MSI-H tumors could benefit from the treatment with a PD-1 blockade because their immune systems are able to recognize a high number of neoantigens, regardless of tumor origin. Moreover, pembrolizumab's robust anti-tumor activity was confirmed via a combined analysis of results from two international phase II trials of pembrolizumab in heavily pretreated patients with dMMR/MSI tumors; the KEYNOTE-164 trial (NCT 02460198) included patients with dMMR/MSI-H mCRC, while the KEYNOTE-158 trial (NCT02628067) included patients with dMMR/MSI-H non-CRC in 27 tumor types[27].

A similar positive effect was observed in the phase II

CheckMate 142 trial (NCT02060188), which was conducted to evaluate the treatment efficacy of nivolumab monotherapy in 74 chemorefractory patients with dMMR/MSI-H mCRC[9]. The estimated rates at 12 months were 50% for PFS and 73% for OS. The treatment response was found to be not associated with a history of Lynch syndrome or the patient's BRAF or KRAS mutation status. Based on the results of these pivotal studies, the United States FDA approved pembrolizumab and nivolumab as the second-line treatment for patients with dMMR/MSI-H mCRC in 2017. The FDA also granted first tumor-agnostic approval to pembrolizumab for dMMR/MSI-H tumors in May 2017. Meanwhile in Japan, pembrolizumab was approved for MSI-H tumors and nivolumab for MSI-H mCRC in December 2018 and February 2020, respectively.

Recently, the clinical benefits of pembrolizumab versus standard chemotherapy as a first-line treatment in 307 patients with dMMR/MSI-H mCRC were demonstrated in the international, randomized phase III KEYNOTE-177 trial (NCT02563002), with PFS and OS as the primary co-endpoints[5]. Treatment with pembrolizumab resulted in doubling PFS, compared with that of chemotherapy (median = 16.5 months vs. 8.2 months; hazard ratio [HR], 0.60; 95% confidence interval [CI], 0.45-0.80; P = .0002). ORRs were determined to be significantly higher with pembrolizumab than with chemotherapy (44% vs. 33%); furthermore, a CR was observed in 11% of patients receiving pembrolizumab,

Table 3. Treatment-related Adverse Events (≥ Grade 3).

	KEYNOTE-177		CheckMate 142	
Treatment line		1st	≥ 2nd	≥ 2nd
Number of patients	153	154	74	119
Regimen	Pembro	Chemo	Nivo	Nivo + Ipi*
Any TRAE	22%	66%	20%	32%
Diarrhea	2%	10%	1%	2%
Fatigue	2%	9%	1%	2%
Nausea	0%	2%	0%	1%
Stomatitis	0%	4%	1%	1%
Neutropenia	0%	15%	-	-
Hepatitis	3%	0%	1%	11%
Colitis	3%	0%	1%	-
Hypothyroidism	0%	0%	0%	1%
Hyperthyroidism	0%	0%	0%	0%
Endocrine	1%	0%	1%	5%
Skin	1%	1%	1%	4%
Pulmonary	0%	0%	0%	1%

Abbreviations: Pembro, pembrolizumab; Nivo, nivolumab; Ipi, ipilimumab; Chemo, chemotherapy;

* Nivo 3 mg/kg + Ipi 1 mg/kg every 3 weeks (four doses), followed by Nivo 3 mg/kg biweekly

compared with 3.9% of those receiving chemotherapy. Notably, 83% of pembrolizumab responders were still responding after 2 years or longer, compared to the 35% of the chemotherapy responders. The rates of Grade 3-5 treatment-related adverse events (AEs) were 22% for pembrolizumab and 66% for chemotherapy (Table 3). The treatment regimens had considerably different toxicity profiles: immune-related AEs (e.g., colitis and hepatitis) with pembrolizumab and traditional AEs (e.g., neutropenia, diarrhea, fatigue, stomatitis, and nausea) with chemotherapy. Based on these findings, which demonstrate pembrolizumab's superiority over chemotherapy with clinically significant improvement in PFS and favorable treatment-related AEs, the FDA has issued its approval for pembrolizumab as the first-line treatment of patients with dMMR/MSI-H mCRC in June 2020. Neither the Japanese Pharmaceuticals and Medical Devices Agency nor the European Medicines Agency has approved pembrolizumab as the frontline regimen. The OS data, another co-primary endpoint, are not yet mature. However, over 60% of patients treated with standard chemotherapy received pembrolizumab or another anti-PD-1/PD-L1 antibody in the second-line setting, and the high cross-over rate will likely affect the survival difference between treatment groups. Notably, pembrolizumab can be administered via a 1-h infusion every 3 weeks, whereas the administration schedule for chemotherapy is a bit more complex. Considering its convenient administration protocol and favorable toxicity profiles, pembrolizumab will likely be the optimal first-line treatment for patients with dMMR/MSI-H mCRC.

2.4. Dual blockade of PD-1 and CTLA-4

Although the KEYNOTE-177 trial demonstrated pembrolizumab's durable clinical benefits in patients with dMMR/MSI-H mCRC, 30% of patients treated with pembrolizumab had primary resistance[5]. Therefore, additional therapeutic strategies are needed in the field of immunotherapy.

Currently, the most promising strategy has been identified to be the dual blockade of PD-1 and CTLA-4. CTLA-4 acts early in the immune response process by inhibiting T-cell activation, whereas PD-1 acts in later stages by turning off anti-tumor T-cell responses[28]. Therefore, dual inhibitors synergistically promote an anti-tumor immune response by blocking complementary mechanisms. The phase II CheckMate-142 trial included a cohort of 119 pretreated patients with dMMR/MSI-H mCRC who received nivolumab and ipilimumab, an anti-CTLA-4 IgG1 monoclonal antibody[8]. Indirect comparisons of nivolumab plus ipilimumab cohort with the nivolumab monotherapy cohort revealed promising results for nivolumab plus ipilimumab: ORR, 55% vs. 31%; 12-month PFS rate, 71% vs. 50%; 12-month OS rate, 85% vs. 73% (Table 1). Based on these results, in July 2018, the FDA has issued approval for the combination treatment with nivolumab plus ipilimumab for dMMR/MSI-H mCRC patients who have progressed after therapy with fluoropyrimidines plus irinotecan or oxaliplatin. However, since combined treatment with nivolumab and ipilimumab versus nivolumab monotherapy resulted in an increased rate of treatment-related AEs (Grade 3 to 4 AEs = 32% vs.

20%), careful management will be required (Table 3).

Promising preliminary results were obtained when nivolumab and ipilimumab were combined in the first-line treatment of 45 patients with MSI-H/dMMR mCRC in the CheckMate 142 trial[29]. In total, an ORR of 60% and a CR of 7% were observed. The 12-month PFS and OS rates were 77% and 83%, respectively. Thus, nivolumab plus ipilimumab may represent a new treatment option in the first-line setting. The international, randomized, phase III CheckMate 8HW trial (NCT04008030), which has been designed to evaluate the efficacy of nivolumab monotherapy, nivolumab plus ipilimumab, or chemotherapy for patients with dMMR/MSI-H mCRC, is still ongoing[30].

Unlike patients with dMMR/MSI-H, combining CTLA-4 and PD-L1 inhibitors showed a limited clinical benefit in patients with non-selected mCRC (Table 2)[31,32]. Thus, a better understanding of the molecular mechanisms involved in immunogenicity in pMMR/MSS CRC is needed to develop predictive biomarkers and effective therapeutic combination strategies.

3. Potent Therapeutic Strategies

As described in the previous section, most CRC patients fail to respond to ICIs due to poor TILs and immunogenicity. Therefore, several treatment strategies have been examined to turn immunologically "cold" tumors with poor immune activation into "hot" tumors with strong immune infiltration in clinical trials combining the anti-PD-1/PD-L1 antibody with other immune-modulating treatments, including other ICIs, angiogenetic inhibitors, molecular-targeted agents, and chemotherapy (Table 2).

3.1. Anti-angiogenetic inhibitors

VEGF has been identified to exert immunosuppressive effects via several mechanisms, such as by decreasing the number of TILs, activating immune checkpoint molecules, inhibiting dendritic cell (DC) differentiation, and downregulating MHC[33]. Anti-angiogenic agents, therefore, could have immunomodulatory effects when combined with an ICI.

In a phase Ib trial (NCT01633970) that involves the combination of the anti-PD-L1 monoclonal IgG1 antibody atezolizumab and the anti-VEGF antibody bevacizumab with or without chemotherapy, the atezolizumab plus bevacizumab treatment had an ORR of 7% and a DCR of 64%, respectively, in 14 patients with refractory pMMR/MSS mCRC[34]. In the randomized, placebo-controlled phase II BACCI trial (NCT0287319) of capecitabine plus bevacizumab with or without atezolizumab in 133 patients with refractory mCRC, adding atezolizumab has prolonged the median PFS (4.4 months vs. 3.3 months) and reached the prespecified primary endpoint, supporting the dual blockade of

PD-1/PD-L1 and VEGF axes[35]. However, in the randomized phase II MODUL trial, which used an umbrella design for biomarker-driven maintenance therapy following first-line treatment with FOLFOX plus bevacizumab in mCRC (NCT02291289), the primary endpoint, PFS, was not reached after atezolizumab was added to 5-FU plus bevacizumab[36]. Currently, several trials evaluating the efficacy of combining ICIs with bevacizumab plus chemotherapy are still ongoing, including the randomized phase III COMMIT trial (NRG-GI004/SWOG-S1610, NCT02997228) of bevacizumab plus mFOLFOX6 with or without atezolizumab or atezolizumab monotherapy in the first-line treatment of patients with dMMR mCRC[37] and the randomized phase II AtezoTRIBE trial (NCT03721653) of bevacizumab plus FOLFOXIRI with or without atezolizumab as the first-line treatment of patients with mCRC, irrespective of MSI status[38].

Regorafenib has been determined as a potent inhibitor of angiogenic and oncogenic kinases, which has been shown to modulate anti-tumor immunity by reducing tumor-associated macrophages[39]. The phase Ib REGONIVO trial (NCT 03406871) of regorafenib plus nivolumab in patients with mCRC showed remarkable anti-tumor activity, coupled with an ORR of 9 (36%) in 25 CRC patients, including 1 dMMR/MSI-H patient, and the median PFS was 7.9 months[40]. However, in the phase II REGOMUNE trial (NCT03475953) of regorafenib plus an anti-PD-L1 IgG1 monoclonal antibody, avelumab, in the treatment of solid tumors, including 48 patients with pMMR/MSS mCRC, no objective response was noted, with a median PFS of 3.6 months in mCRC[41]. Furthermore, a phase I/IB trial of regorafenib plus nivolumab in 28 patients with refractory pMMR/MSS CRC showed similar results as the REGOMUNE trial, with an ORR of 5% and a median PFS of 4.3 months[42]. Thus, combination treatment with regorafenib and ICIs can result in modest clinical activity in patients with pMMR/MSS CRC.

3.2. Chemotherapy and radiotherapy

It has been shown that chemotherapy and radiotherapy (RT) could cause immunogenic cell death (ICD) in tumor cells, which is recognized by DC and activates CD8+ T-cells[43]. Therefore, strategies combining ICIs with chemotherapy or RT may pave the way in overcoming primary resistance to immune therapy in patients with mCRC. The preclinical data demonstrate that 5-fluorouracil (5-FU) treatment enhances TILs and the anti-tumor immune response by eliminating myeloid-derived suppressor cells (MDSCs)[44]. Since platinum-based oxaliplatin may also induce ICD[45], combining FOLFOX (5-FU plus oxaliplatin) with ICIs may also be a promising treatment regimen. Phase Ib/II of the single-arm MEDETREME trial (NCT03202758) has investigated an anti-PD-L1 IgG1 kappa monoclonal antibody, dur-

valumab, and an anti-CTLA-4 IgG2 monoclonal antibody, tremelimumab, in combination with FOLFOX, in the first-line treatment of patients with RAS-mutated MSS mCRC. The intermediate analysis showed that this regimen has great treatment potential, with an ORR of 63%, CR of 31%, and a DCR of 63%[46]. In a cross-sectional study of 98 clinical trials testing ICIs alone or in combination with other agents, the most compatible partner of ICIs would be the platinum chemotherapy or an anti-angiogenetic inhibitor, supporting combination treatment utilizing an angiogenetic inhibitor and platinum chemotherapy with ICIs[47]. Currently, as described in the previous section regarding anti-angiogenetic inhibitors, several trials using such treatment strategy are ongoing: the phase III COMMIT trial[37] and the phase II AtezoTRIBE trial[38].

RT then enhances the diversity of the TCR repertoire of intra-tumoral T-cells via DNA damage and is drawing attention from the field of immunotherapy[48]. Several trials have demonstrated synergistic effects between RT and ICIs in patients with non-small cell lung cancer (NSCLC)[4,49], indicating the rationale for this combinational strategy. However, the amount of data available regarding the use of this strategy in mCRC has remained limited. A pilot study (NCT 02298946) examining the combination of a PD-L2 Fc fusion protein, AMP-224, with stereotactic body radiation therapy in patients with mCRC has found no significant clinical benefits[50]. In a single-arm phase II trial (NCT02437071) assessing the efficacy of pembrolizumab plus RT or ablation in patients with pMMR/MSS, 1 in 11 patients in the RT cohort had an objective response in a metastatic site distant from the irradiation field[51]. Additionally, the optimal conditions for RT, including timing, dose fractionation, and the irradiation field, remained to be unclear[52].

Patients diagnosed with rectal cancer could be candidates for a combined treatment with RT and ICIs because preoperative chemoradiation (CRT) is one of the standard treatments in rectal cancer and upregulates PD-L1 expression[53,54]. In the phase I/II investigator-initiated VOLT-AGE trial (NCT02948348) of nivolumab monotherapy and subsequent radical surgery following preoperative CRT in patients with MSS locally-advanced rectal cancer, 11 (30%) and 14 (38%) of 37 patients with MSS were CR and major responses by pathological examination, respectively, suggesting their potential for future use in non-operative management[55].

3.3. T-Cell bispecific antibody therapy

The T-cell bispecific antibody (TCB) has been utilized in a new anti-tumor immunotherapeutic approach that involves engineering TCBs to facilitate T-cells' engagement with tumor cells. By taking advantage of carcinoembryonic antigen (CEA) overexpression, which is frequently observed on the cell surface in most mCRC cells, CEA-TCB simultaneously binds to CEA on tumor cells and to CD3 on T-cells, thus attacking tumor cells independently of the neoantigen load, pre-existing immunity, and TILs. In the phase I trials of CEA-TCB (RG7802 and RO6958688) as a single agent (NCT02324257) and in combination with the anti-PD-L1 atezolizumab (NCT02650713) in CEA-positive solid tumors, the preliminary clinical data from mCRC showed an ORR of 6% in monotherapy and 20% in combination therapy. Furthermore, all patients who experienced a partial response were MSS CRC, suggesting a potent immunotherapy agent, especially in combination with ICIs, for poorly immunogenic dMMR/MSS CRCs[56]. However, this treatment has been observed to induce more adverse effects, with higher rates of pyrexia, infusion-related reactions, and diarrhea.

3.4. Inhibitory immune checkpoints

The PD-1/PD-L1 interaction is not the only immune checkpoint pathway regulating T-cell activation in the tumor microenvironment. T-cell immunoglobulin mucin receptor 3 (TIM3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), and lymphocyte activation gene 3 protein (LAG3) are overexpressed on effector CD4+ and CD8+ T-cells, regulatory T-cells (Tregs), and natural killer (NK) cells; furthermore, they act as inhibitory immune checkpoint modulators[10,57,58].

TIM-3 binds primarily to galectin-9, which triggers T-cell apoptosis and negatively regulates the Th1 response in the induction of peripheral tolerance[59]. In immunocompetent mouse models of lung adenocarcinoma, tumors progressing after responding to a PD-1 inhibitor have exhibited upregulation of TIM-3 on PD-1 antibody bound T-cells, and an additional survival benefit of TIM-3 inhibition following PD-1 blockade failure was observed[58]. In another preclinical model, the TIM-3 inhibitor alone showed modest therapeutic activity, but the combined blockade of TIM-3 with CTLA-4 and PD-1 resulted in remarkable tumor regression[60]. These findings suggest that TIM-3 may be a targetable molecule.

TIGIT binds to CD155 with high affinity and competes with its activating counter-receptor CD226, which suppresses anti-tumor immunity through its expression not only on Tregs but also on CD8+ T-cells and NK cells[58]. Since tumor-infiltrating CD8+ T-cells exhibit co-expression of other inhibitory checkpoint molecules, such as PD-1, TIM-3, and LAG-3, the dual blockade of TIGIT and these inhibitory molecules synergistically enhances anti-tumor activity in the syngeneic CRC model[61]. In the randomized phase II CITYSCAPE trial (NCT03563716), which evaluated the efficacy and safety of the anti-TIGIT monoclonal antibody tiragolumab (also known as MTIG7192A and RG6058) plus the anti-PD-L1 atezolizumab compared with atezolizumab alone as a first-line treatment for patients with PD-L1-positive NSCLC, tiragolumab and atezolizumab showed

clinically meaningful improvements in the ORR and PFS when compared to placebo plus atezolizumab[62].

LAG-3 is structurally homologous to CD4; it has high-affinity binding to MHC class II, inducing the activation of Tregs and suppression of CD8+ T-cells and DC[63]. Two inhibitory immune molecules, LAG-3 and PD-1, synergistically regulate T-cell function in promoting immune escape, guiding the dual blockade of these inhibitory molecules[64]. Blocking these molecules may lead to a less exhausted phenotype and further activate anti-tumor immunity; currently, several trials assessing their efficacy are ongoing for various solid tumors, including CRC[57].

3.5. Stimulatory immune checkpoints

In contrast to blockading inhibitory immune molecules, the agonist antibodies of stimulatory molecules belonging to the tumor necrosis factor (TNF) receptor superfamily, including OX40 (also known as CD134), CD40, the glucocorticoid-induced TNF receptor-related gene (GITR; also known as CD357), and 4-1BB (also known as CD137), may also be deemed beneficial in activating T-cell functions[57]. OX40 has been found to be expressed on all T-cell subsets, whereas its ligand OX40L is expressed on APCs. Interaction between OX40 and OX40L stimulates the T-cell response, expansion of memory T-cells, cytokine production, depletion of Tregs, and activation and maturation of DCs[65]. The potent efficacy of its agonist antibody was demonstrated in several preclinical models[66]. Similar to OX40, GITR also impairs the infiltration and inhibitory function of Tregs[67].

4-1BB has been identified to promote cell proliferation, survival, and cytokine production through nuclear factor (NF)-κB and the MAPK pathways[68]. Although a single agent of the 4-1BB agonist antibody showed limited immune activity in a phase I trial (NCT01307267)[69], combination treatment of PD-1 antagonist and 4-1BB agonist resulted in pronounced tumor inhibition, dependent on IFN-γ and CD8+ T-cells, in a poorly immunogenic melanoma model[70].

CD40 promotes the upregulation of MHC class II on APCs and the secretion of proinflammatory cytokines, which elicit the CD8+ T-cell response[71]. Unfortunately, previous clinical trials found that CD40 agonists have limited monotherapy efficacy[72]. However, a recent single T-cell analysis by RNA sequencing and TCR tracking has revealed the rapid expansion of basic helix-loop-helix family member E40 (BHLHE40)+ Th1 like CD4+ T-cells, similar to the immune phenotype of MSI-H tumors, following treatment with a CD40 agonist[73]. Thus, CD40 agonist antibodies may be able to convert immunologically "cold" tumors into "hot" tumors and further induce a sensitive response to PD-1/PD-L1 inhibitors.

3.6. Transforming growth factor-β (TGF-β)

The TGF-β pathway in fibroblasts has been determined to contribute to excluding CD8+ T-cells from the tumor parenchyma, which in turn attenuates the response to a PD-L inhibitor. A dual blockade of TGFβ and PD-L1 facilitates T-cell infiltration into the center of a tumor and subsequent vigorous anti-tumor immunity via reduced TGF-β signaling in stromal cells[74]. Based on this evidence, a combined TGF-β and PD-1/PD-L1 inhibitor is currently under evaluation in clinical trials in solid tumors[75]. In an expansion cohort of a phase I trial (NCT02517398) of a bifunctional fusion protein targeting PD-L1 and TGF-β, M7824 (MSB 0011359C), for patients with mCRC, only 1 patient in 29 evaluable patients with CRC had a confirmed objective response, and this patient was MSS and consensus molecular subtype (CMS) 4[76]. Since CMS4 tumors are characterized by the marked upregulation of TGF-β signaling[77], patients with CMS4 may benefit from a dual blockade of PD-L1 and TGF-β.

3.7. Epidermal growth factor receptor (EGFR)

An anti-EGFR IgG1 chimeric mouse-human monoclonal antibody, cetuximab, has been considered as one of the standard treatments in patients with RAS wild-type CRC. Preclinical data have shown that treatment with cetuximab stimulates the opsonization and phagocytosis of colon cancer cells by DC and antibody-dependent cellular cytotoxicity, which promotes immune response activation[33,78]. In the single-arm, exploratory phase II AVETUX trial (AIO-KRK-0216, NCT03174405), which examined a combination treatment of anti-PD-L1 avelumab with FOLFOX plus cetuximab as the first-line approach in patients with RAS and BRAF wild-type mCRC, including 95% MSS, a novel treatment response was observed, having an ORR of 80% and DCR of 92%[79]. The ongoing single-arm phase II CAVE trial (EudraCT number: 2017-004392-32) is evaluating a re-challenge strategy with an anti-EGFR antibody, in combination with avelumab, in patients with RAS wild-type mCRC who had an objective response from first-line treatment with chemotherapy plus an anti-EGFR antibody[80].

3.8. Mitogen-activated protein kinase kinase (MEK)

Preclinical data have shown that MEK inhibition results in IFN-γ-dependent MHC upregulation, PD-L1 overexpression, and synergistic tumor regression when combined with PD-1 inhibition[81]. However, the randomized phase III IMblaze370 trial (NCT02788279) has failed to demonstrate statistically significant prolonged OS after treatment with an anti-PD-L1, atezolizumab, with or without the MEK inhibitor cobimetinib, compared to regorafenib in refractory patients with pMMR/MSS CRC[82]. The ORRs were 2%-3% in all treatment arms, and treatment efficacy was not found

to be associated with the subjects' RAS mutation or PD-L1 expression status.

3.9. Other molecules

Tumor-associated macrophages (TAMs) are considered attractive targets to complement PD-1/PD-L1 inhibitors. Interaction between the colony-stimulating factor 1 (CSF1) ligand and CSF1 receptor (CSF1R) regulates the survival of TAMs, which act as a key orchestrator of the immunosuppressive tumor microenvironment[83]. In CRC, secreted phosphoprotein 1 (SPP1)-positive TAMs are resistant to indiscriminate depletion by an anti-CSF1R inhibitor; therefore, specific eradiation of SPP1+ TAMs may lead to improved immunotherapy outcomes[84]. Indolamine-2,3-dioxygenase 1 (IDO-1) is an enzyme that catalyzes the conversion of tryptophan into kynurenine, triggering immune tolerance via the suppression of T-cell functions and activation of Treg[85]. The stimulator of interferon genes (STING), which is the endoplasmic reticulum protein, pathway is activated in APCs within the tumor microenvironment, subsequently driving T-cell priming via type I interferon signaling[86]. Furthermore, DNA damage responses mediated through DNA-damaging chemotherapy or the loss of DNA repair function can also induce STING activation and antitumor immunity[75]. Since blockading these molecules has shown minimal monotherapy efficacy, combination of these molecules inhibitor with ICIs or chemotherapy might be able to potentiate the effects of tumor immunotherapy.

4. Neoadjuvant and Adjuvant Treatments with Immunotherapy

A growing body of evidence suggests the implementation of a consensus Immunoscore, based on CD3+ and CD8+ T-cell densities within the tumor, as an independent prognostic biomarker in patients with early-stage CRC[87]; this raises the question of whether patients with a high immune phenotype may receive a potent benefit from immunotherapy in a neoadjuvant or adjuvant setting for early-stage CRC. In early-stage NSCLC, a pilot study (NCT02259621) of neoadjuvant therapy with nivolumab showed a major pathological response of 45% with few AEs, and no incidences of delayed surgery were determined[88]. Thus, immunotherapy may be highly effective in patients with early-stage cancer. In the exploratory NICHE study (NCT03026140) of neoadjuvant treatment with two doses of nivolumab plus a single dose of ipilimumab in patients with dMMR or pMMR tumors, the pathological response rate was 100% in 20 patients with dMMR and 27% in 15 patients with pMMR. Notably, pathological CR was 60% in dMMR, but none was observed in pMMR[89]. These impressive results suggest a potential paradigm shift to neoadjuvant immunotherapy or non-operative management in patients with early-stage

dMMR/MSI-H or with a subset of pMMR/MSS CRC.

Adjuvant chemotherapy is considered a standard treatment in patients with stage III CRC[2]. Only 5% of patients with mCRC have dMMR/MSI-H tumors, but this phenotype is present in 12% of patients with Stage III CRCs[18]. Currently, two phase III randomized trials are ongoing to assess the efficacy of immunotherapy in the adjuvant setting. The ATOMIC trial (NCT02912559) is evaluating the combination of FOLFOX plus anti-PD-L1 atezolizumab vs. FOLFOX as an adjuvant treatment in 700 patients with Stage III MSI-H CRC[90]. The POLEM trial (NCT03827044) is also assessing the role of an anti-PD-L1, avelumab, as maintenance treatment after 5-FU-based adjuvant chemotherapy in 402 patients with Stage III MSI-H or the proofreading exonuclease activity intrinsic to replicative DNA polymerases epsilon (POLE)-mutant CRC[91].

5. Predictive Biomarkers for ICIs

A growing body of evidence suggests that a patient's dMMR/MSI-H status is a robust predictive biomarker for the treatment with ICIs in CRC. However, some patients with MSI-H CRC do not benefit from treatment with ICIs. Additionally, approximately 95% of patients with mCRC are pMMR/MSS, highlighting the need to identify more precise and reliable predictive biomarkers for ICIs. Currently, various biomarkers of response to ICIs are being explored.

One emerging biomarker response to ICIs is the tumor mutational burden (TMB), defined as the total number of mutations per coding area of a tumor genome[92,93]. In a 10-cohort phase II KEYNOTE-158 trial of pembrolizumab for previously treated patients with advanced non-CRC tumors, the efficacy of TMB-H (≥10 mutations/megabase) was assessed as a prospectively planned retrospective analysis[94]. TMB-H was detected in 99 patients via FoundationOne CDx™ assay, and the ORR was 30% with a CR of 4%, compared with an ORR of 6.7% in 652 patients with non-TMB-H. Notably, the ORR was 27% in patients with TMB-H, excluding MSI-H. The 12-month PFS rates were 26.4% for TMB-H and 14.1% for those with non-TMB-H[94]. Based on these findings, in June 2020, the FDA granted pembrolizumab the second tumor-agnostic approval for heavily treated patients with TMB-H tumors, following the first approval for patients with dMMR/MSI-H tumors. However, these findings were based on data collected from a few patients without including CRC, and the appropriate threshold for defining TMB-H as a predictive biomarker should be dependent on the tumor type rather than universal across a wide variety of tumor types[92]. dMMR/MSI-H mCRC patients have a better response to ICIs than would be predicted by the status of TMB, whereas in patients with pMMR/MSS, the response is worse than would be predicted[93]. Thus, a considerable amount of research is

needed to assess and validate TMB-H in patients with mCRC.

The POLE is critical in the maintenance of DNA replication fidelity and prevention of mutagenesis. POLE somatic mutations are mutually exclusive with dMMR, ranging from 1% to 3% of CRCs[12,95]. Interestingly, POLE mutant CRCs confer a remarkably hypermutated somatic profile, increased CD8+ TILs, and high expression of immune checkpoint molecules, similar to dMMR CRCs' clinical-pathological features[77,95]. Given the ultra-mutated and immunogenic phenotypes, a POLE mutation would have the therapeutic potential of ICIs, even in patients with pMMR/MSI CRCs[96].

Immunoscores have been used to stratify prognostic outcomes in patients with early-stage CRC[87]. Furthermore, a T-cell-inflamed gene expression profile and TMB are determined to be independently predictive of PD-1 inhibitor responses because they capture distinct features of T-cell activation and immunogenicity, indicating the potential of these combinations in identifying responders and non-responders to ICIs[97]. Thus, immune profiles of the tumor microenvironment might provide a clue in predicting the response to an ICI[15].

Since neoantigens are produced by tumor-somatic mutations, tumor cells with high TMB generally display a high neoantigen load, which confers tumor immunogenicity and can elicit a tumor immune response[10,98]. A recent study revealed that only a few of the products encoded by somatic nonsynonymous mutations are immunogenic[98]. Thus, the lack of optimal methods to assess neoantigens as a predictive marker is among the main issues.

From the theoretical background of PD-1/PD-L1 inhibitors, PD-L1 expression was among the candidate predictors of the response to a PD-1 blockade in several tumor types, such as NSCLC[99]. However, in several studies of CRC patients treated with ICIs, no significant difference was noted in treatment efficacy, based on the level of PD-L1 expression[7-9,25]. For instance, in the phase II CheckMate 142 trial, treatment response of PD-L1 positive and negative expression was similar in dMMR/MSI-H mCRC patients treated with nivolumab[9]. Moreover, certain commensal microbiota are likely to be associated with the efficacy of ICIs[100]. In summary, TMB-H, POLE mutations, immune profiles, neoantigens, and the microbiome are promising predictive biomarkers for treatment with ICIs, but further research and validation are needed.

6. Conclusion

ICI-based immunotherapy has revolutionized anti-tumor treatment in various tumor types in recent years. However, this approach is currently available for patients with dMMR/MSI-H mCRC, who represent only 5% of mCRCs. There-

fore, strategies to render pMMR/MSS tumors a similar phenotype to dMMR/MSI-H tumors are arguably needed. Several clinical trials are ongoing to assess the treatment efficacy of combining PD-1/PD-L1 inhibitors with other ICIs, chemotherapy, RT, angiogenetic agents, and molecular-targeted agents. An in-depth understanding of the complexity and diversity of the immune system's functioning within the tumor microenvironment will increase the likelihood of developing predictive biomarkers and novel therapeutic strategies to potentiate anti-tumor immunity in patients with CRC.

Author Contributions

A.O. searched the literature and wrote the manuscript. E.S. and K.Y. drew conceptual frameworks and revised the manuscript.

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