

# World Journal of *Gastrointestinal Surgery*

*World J Gastrointest Surg* 2023 April 27; 15(4): 495-744



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The primary aim of *World Journal of Gastrointestinal Surgery* (*WJGS, World J Gastrointest Surg*) is to provide scholars and readers from various fields of gastrointestinal surgery with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

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**INDEXING/ABSTRACTING**

The *WJGS* is now abstracted and indexed in Science Citation Index Expanded (SCIE, also known as SciSearch®), Current Contents/Clinical Medicine, Journal Citation Reports/Science Edition, PubMed, PubMed Central, Reference Citation Analysis, China National Knowledge Infrastructure, China Science and Technology Journal Database, and Superstar Journals Database. The 2022 Edition of Journal Citation Reports® cites the 2021 impact factor (IF) for *WJGS* as 2.505; IF without journal self cites: 2.473; 5-year IF: 3.099; Journal Citation Indicator: 0.49; Ranking: 104 among 211 journals in surgery; Quartile category: Q2; Ranking: 81 among 93 journals in gastroenterology and hepatology; and Quartile category: Q4.

**RESPONSIBLE EDITORS FOR THIS ISSUE**

Production Editor: Rui-Rui Wu, Production Department Director: Xiang Li, Editorial Office Director: Jia-Ru Fan.

**NAME OF JOURNAL**

*World Journal of Gastrointestinal Surgery*

**ISSN**

ISSN 1948-9366 (online)

**LAUNCH DATE**

November 30, 2009

**FREQUENCY**

Monthly

**EDITORS-IN-CHIEF**

Peter Schemmer

**EDITORIAL BOARD MEMBERS**

<https://www.wjgnet.com/1948-9366/editorialboard.htm>

**PUBLICATION DATE**

April 27, 2023

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**INSTRUCTIONS TO AUTHORS**

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**PUBLICATION ETHICS**

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**ARTICLE PROCESSING CHARGE**

<https://www.wjgnet.com/bpg/gerinfo/242>

**STEPS FOR SUBMITTING MANUSCRIPTS**

<https://www.wjgnet.com/bpg/GerInfo/239>

**ONLINE SUBMISSION**

<https://www.f6publishing.com>



## Current and emerging therapeutic approaches for colorectal cancer: A comprehensive review

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**Specialty type:** Gastroenterology and hepatology

**Provenance and peer review:** Invited article; Externally peer reviewed.

**Peer-review model:** Single blind

**Peer-review report's scientific quality classification**

Grade A (Excellent): 0  
Grade B (Very good): 0  
Grade C (Good): C, C, C  
Grade D (Fair): 0  
Grade E (Poor): 0

**P-Reviewer:** Luo W, China; Wang LH, China

**Received:** October 21, 2022

**Peer-review started:** October 21, 2022

**First decision:** January 3, 2023

**Revised:** January 11, 2023

**Accepted:** March 3, 2023

**Article in press:** March 3, 2023

**Published online:** April 27, 2023



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### Abstract

Colorectal cancer (CRC) affects 1 in 23 males and 1 in 25 females, making it the third most common cancer. With roughly 608000 deaths worldwide, CRC accounts for 8% of all cancer-related deaths, making it the second most common cause of death due to cancer. Standard and conventional CRC treatments include surgical expurgation for resectable CRC and radiotherapy, chemotherapy, immunotherapy, and their combinational regimen for non-resectable CRC. Despite these tactics, nearly half of patients develop incurable recurring CRC. Cancer cells resist the effects of chemotherapeutic drugs in a variety of ways, including drug inactivation, drug influx and efflux modifications, and ATP-binding cassette transporter overexpression. These constraints necessitate the development of new target-specific therapeutic strategies. Emerging therapeutic approaches, such as targeted immune boosting therapies, non-coding RNA-based therapies, probiotics, natural products, oncolytic viral therapies, and biomarker-driven therapies, have shown promising results in preclinical and clinical studies. We tethered the entire evolutionary trends in the development of CRC treatments in this review and discussed the potential of new therapies and how they might be used in conjunction with conventional treatments as well as their advantages and drawbacks as future medicines.

**Key Words:** Colorectal cancer; Chemotherapy; Immunotherapy; RNA interference; Probiotics; Oncolytic viral therapy

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**Core Tip:** This review highlights some of the latest colorectal cancer treatment approaches that have the potential to soon translate into standard care. A vast literature of patient data has revealed that conventional therapies are non-specific and have numerous secondary complications. Additionally, patients develop resistance to conventional chemotherapies. There is a need to develop new target-specific arrows in the cancer-fighting quiver.

**Citation:** Kumar A, Gautam V, Sandhu A, Rawat K, Sharma A, Saha L. Current and emerging therapeutic approaches for colorectal cancer: A comprehensive review. *World J Gastrointest Surg* 2023; 15(4): 495-519

**URL:** <https://www.wjgnet.com/1948-9366/full/v15/i4/495.htm>

**DOI:** <https://dx.doi.org/10.4240/wjgs.v15.i4.495>

## INTRODUCTION

Starting from the industrial revolution, the scientific world has evolved progressively. After reaching the 21<sup>st</sup> century, certain diseases need novel therapeutic moieties for their cure, and colorectal cancer (CRC) is one of them. CRC is the third most prevalent cancer globally (6.1%) after lung cancer (11.6%) and breast cancer in females (11.6%) and prostate cancer in males (7.1%)[1]. It ranks second among all cancer in terms of mortality, accounting for 9.2% of all cases (9% male and 8% female)[1]. Estimates have shown that by the year 2035, there will be an increment in the cases of colon and rectal cancer by 71.5% and 60.0%, respectively[2]. This rapid disease progression has led to an economic burden on the countries and requires a major part of gross domestic product expenditure on public health.

The primary therapy for resectable CRC is surgical removal, and in non-resectable CRC, standard therapies include chemotherapy, radiotherapy, and immunotherapy. However, these therapies have certain drawbacks, such as being non-specific and cytotoxic to normal cells, which leads to secondary complications[3]. Depending upon the confinement and progression of the CRC, these therapies can be utilized in combinations. However, even with combinational therapies, more than half of patients relapse into acquired multidrug resistance CRC[4]. According to 2021 statistics, despite significant advancements in CRC screening, surgical resection, and adjuvant treatment, the death rate for CRC patients is still relatively high[1]. Therefore, there is a need to develop novel CRC therapies that render resistant tumors more sensitive to chemotherapeutic drugs.

Immune checkpoint inhibitors (ICIs), chimeric antigen receptor (CAR) T cell therapy, T cell receptor (TCR) alterations, and cytokine therapy have recently emerged as effective treatments for CRC. Also recent research on the use of probiotics[5], RNA-based therapies [small interfering RNA (siRNA), microRNA (miRNA), and RNA aptamer][6], oncolytic viral therapies[7], and natural products[8] in the treatment of CRC have yielded promising results. However, the survival rate of patients at an advanced stage remains a major problem. Scientists are working hard to understand the pathophysiology of CRC to have a better approach for treatment while enhancing current treatments like radiation therapy, targeted therapy, endoscopic resection, chemotherapy, and immunotherapy.

An individualized standard chemotherapeutic regimen is prescribed to a patient based on certain factors such as overall health profile, co-medication, comorbidities, patient compliance, psychosocial factors, results of prior resection, adjuvant therapy, route of administration, logistical support, consideration of side effect profiles, the biology of the tumor, the main location of the tumor, the presence of *RAS* and *BRAF* mutations, or microsatellite instability. Considering these factors, patients are divided into risk groups with varying treatment strategies. Since colon cancers and rectal cancers are different, multidisciplinary and distinct approaches must be taken depending on the staging information before treatment[9]. This review traced the evolutionary development of many standard CRC treatments and recent promising therapeutic approaches. We also reviewed the benefits and hurdles that still need to be overcome in treating CRC.

## THERAPEUTIC APPROACHES

### Local approaches

**Radiation therapy:** Neoadjuvant therapy, including radiotherapy and chemotherapy when used alone or in combination, has been recommended in rectal cancer and has effectively reduced tumor burden for intermediate and advanced stage cancer. The primary objective of radiotherapy is to decrease the risk of local recurrence and improve overall survival. However, evidence suggests that preoperative radiotherapy is more effective than postoperative therapy in reducing local recurrence, but it does not improve overall survival[10]. With two available adjuvant radiotherapies, short-course radiotherapy (RT) and long-course RT, there was always a question of which one was better. Higher rates of acute

toxicity are observed with long-course RT than with short-course RT, but late side effect incidence rates do not show significant differences[11]. Radiation and adjuvant radiation therapy are better options for treating stage II and III CRC. However, radiation therapies have some plausible long-term toxicity impacts on vital organs.

New delivery methods, such as intensity-modulated RT (IMRT), have been widely adopted in clinical practice and have demonstrated potential benefits with decreased toxicity for patients with rectal cancer by lowering the radiation dose[12]. IMRT employs linear accelerators to safely deliver precise radiation to a tumor while reducing the exposure to nearby healthy tissue. With IMRT, radiation dosages to adjacent healthy organs can be limited while still being delivered at high doses to the tumor and nearby lymph nodes. By adjusting the dose in this way to avoid normal, unaffected tissues, it can lessen adverse effects and potentially improve the toxicity profile. Additionally, the use of IMRT for rectal cancer may help to speed up the time to surgery, promote better postoperative recovery, and enhance the tolerability of adjuvant chemotherapy[13]. IMRT may help reduce treatment interruptions, emergency department visits, and hospitalizations compared to 3-dimensional conformal radiation therapy[14]. In a United Kingdom study of radiotherapy facilities, 68% of respondents reported using IMRT for all rectal cancer patients[15]. In the future, more clinical data will be needed to support the use of IMRT for rectal cancer, which could encourage doctors to incorporate IMRT planning into preoperative chemoradiotherapy for rectal cancer.

### Systemic approach

**Chemotherapy:** The ultimate treatment for locoregional CRC is surgical resection. Advances in primary and secondary treatments have improved the survival time in CRC. Notably, in some circumstances, chemotherapy or radiotherapy may be used as neoadjuvant or adjuvant treatments before or after surgery in order to significantly reduce and cure the tumor. Of note, chemotherapy is used to either eliminate cancer cells or stop them from proliferating. Cytotoxic drugs approved for CRC slow down disease progression and increase an individual's lifespan. The medications approved are fluoropyrimidines, irinotecan, oxaliplatin, tri-fluridine-tipiracil, capecitabine, and 5-fluorouracil (5-FU), most commonly used as a chemotherapeutic agent for curing CRC.

Adjuvant fluoropyrimidine-based chemotherapy after surgery for CRC has been a standard treatment due to their ability to reduce the recurrence of the tumor and increase the survival as shown in trial by Moertel *et al*[16]. Leucovorin (LV, folinic acid), a chemoprotectant, potentiates the activity of 5-FU by forming a stable complex and preventing its adverse effects[17]. The fluorouracil + L-folinic acid (FU/LV) regimen given daily for 5 d in 6 cycles resulted in a 15% reduced risk of death at 5 years[18,19]. All 5-FU-based regimens must include LV as it has been demonstrated to improve patient survival and tumor response rate (RR) when combined with 5-FU[20]. Despite being one of the safest chemotherapeutic agents, 5-FU has side effects for some CRC patients, including fever, mucositis, stomatitis, leukopenia, and thrombocytopenia[21]. Cerebellar ataxia and other neurological diseases also affect 1% of patients[22].

Another imperative drug used is capecitabine, a prodrug of 5-FU. Its bioavailability is virtually 100%, and C<sub>max</sub> and area under the curve increase linearly with dosage. In patients with metastatic CRC, two phase III randomized studies compared capecitabine as a single drug to the typical 5-FU/LV therapy combination, and the RR in both studies were as efficient as 5-FU/LV[23].

Topoisomerase I inhibitor irinotecan and oxaliplatin were added to the 5-FU regimen as part of a cytotoxic combination therapy for metastatic colorectal cancer (mCRC) to enhance its efficacy. Oxaliplatin is a diamminocyclohexane platinum complex that has the potential to generate DNA adducts and interferes with the mechanism of DNA repair, thereby leading to a cytotoxic effect in CRC[22]. Patient survival improved due to the inclusion of oxaliplatin or irinotecan in the 5-FU regimen but at the same time has increased the toxicity levels. In the first-line treatment of mCRC, 5-FU/LV is coupled with oxaliplatin (FOLFOX), irinotecan (FOLFIRI), and both oxaliplatin and irinotecan (FOLFOXIRI). The combination therapies FOLFOX, FOLFIRI, and FOLFOXIRI have established themselves as effective cytotoxic regimens, with an average improvement in survival of about 2 years. According to the Gruppo Oncologico dell'Italia Meridionale trial, overall survival (OS) rates for FOLFIRI and FOLFOX were 14 mo and 15 mo, respectively. The GERCOR trial found that OS was 21.5 mo for patients who received FOLFOX first followed by FOLFIRI and 20.6 mo for those who received FOLFIRI first followed by FOLFOX. Trifluridine-tipiracil, a fluoropyrimidine, contains tipiracil hydrochloride, which prevents the degradation of trifluridine. The incorporation of trifluridine into DNA is the primary mechanism of action. Compared to the best supportive care alone, the prospective randomized clinical phase III trial RECURSE found that it significantly increased median OS[24].

Tyrosine kinase signaling pathways typically prevent unchecked cell growth or boost sensitivity to trigger apoptosis. These signaling pathways are often genetically or epigenetically dysregulated in cancer cells, offering them a selective advantage. The human genome contains about 500 protein kinase genes, which constitute about 2% of all human genes. The structures of over 280 human protein kinases have been determined[25]. Regorafenib is a Food and Drug Administration (FDA)-approved tyrosine kinase inhibitor that targets numerous targets, including vascular endothelial growth factor (VEGF) receptor (VEGFR), platelet-derived growth factor, fibroblast growth factor, and BRAF in mCRC due to its better median OS and progression-free survival (PFS) in the phase III CORRECT trial[26] and the

CONCUR trial[27]. Other promising tyrosine kinase inhibitors are listed in Table 1 with their specific target.

Neoadjuvant chemotherapy (NACT) is an emerging field in cancer. It is mainly used to treat solid tumor malignancies such as gastric, esophageal, and rectal cancers[28], but its efficacy has not been fully explored in cases of CRC. Utilization of NACT before surgery can result in better outcomes in CRC patients with liver metastases. As a result, NACT is being investigated in primary rectal and colon cancers as a potential method to reduce the size of the tumor, enable a curative resection, and mitigate the chance of metastases. A potential benefit of NACT was reported in a trial on patients with stage III colon cancer, in which they received preoperative capecitabine + oxaliplatin (CAPOX) followed by adjuvant therapy (CAPOX) after resection. Tumor volume was reduced by 69.5%, without any progression of disease during therapy, and OS was 100%[29]. A similar protocol with an addition of 5-FU in some cases[30] resulted in the majority of patients experiencing a reduction in tumor volume of 62.5%. This finding was the basis of an ongoing randomized phase II study of neoadjuvant CAPOX in locally advanced colon cancer.

Based on the efficacy of FOLFOXIRI established in mCRC, the same triplet therapy was utilized as NACT in patients with stage IIIB colon cancer, followed by resection and then adjuvant therapy of either FOLFOXIRI or CAPOX. This trial documented a reduction in tumor in 91.3% of patients with toxicities of grade 3-4. At the end of trial, only 52.2% of patients were left with OS of 95.7% and 2-year recurrence rate of 26.1%[31]. There are several other proposed and ongoing randomized phase II and III clinical trials taking place utilizing NACT such as CAPOX, FOLFOX, ipilimumab + nivolumab ± celecoxib and many more[32]. Despite the controversial role of targeted agents in other cancers, these could be considered as a potential weapons in CRC, enhancing the efficacy of neoadjuvant therapy[33]. Treatment with bevacizumab and chemotherapy before surgery in CRC patients with liver metastases has been found to increase the survival in several phase II trials[34]. The efficacy of targeted neoadjuvant therapy requires studies involving larger population. Therefore, definite conclusions cannot be drawn from trials involving small population size, demanding further investigations.

Presently, progress in the adjuvant therapy following resection has taken a pause. A shift has been taken towards the utilization of NACT in both colon and rectal cancers instead. Large, randomized clinical trials are being conducted worldwide to investigate various strategies used for safe and accurate administration of treatment. However, certain parameters like standardization of regimen combination, standard intensity, and duration of therapy, postoperative settings, *etc* are still undefined because of lack of long-term oncological results of randomized phase II and phase III studies, warranting further investigation.

### Target-specific approaches

**Immunotherapy as a promising candidate for CRC treatment:** Following early breakthroughs in the treatment of melanoma, immunotherapy has been quickly established as a prominent therapeutic strategy for a variety of solid tumors[35,36], including CRC. Cancer immunotherapy conquers the issue of specificity, which is a severe issue of chemotherapy, radiotherapy, and other approaches. Figure 1 illustrates all currently available and emerging immunotherapies for CRC. The cancer immunotherapy area has shown potential in treating a variety of solid tumors, with a growing number of FDA-approved monoclonal antibodies and single and combinational immunotherapeutic medicines throughout time [37].

**Anti-epidermal growth factor/receptor antibodies:** The epidermal growth factor receptor (EGFR) signaling pathway is a complicated and closely controlled process that plays a role in proper cell development, proliferation, and survival. When this pathway malfunctions and continues unregulated, neoplastic cells can grow, proliferate, survive, and spread. With encouraging preclinical results, cetuximab, the first monoclonal antibody targeting EGFR, was introduced. Cetuximab is a chimeric immunoglobulin G (IgG) antibody that causes EGFR internalization and destruction[38]. According to the BOND trial, which supported the FDA's 2004 approval of cetuximab for treating mCRC, cetuximab showed considerable promise in improving PFS and OS in patients with a low response to single-agent IRI therapy[39]. Cetuximab, in combination with other chemotherapies, showed encouraging outcomes as well. The phase III CRYSTAL trial found that the combination of cetuximab and the FOLFIRI regimen had better progression control than FOLFIRI alone [8.9 mo *vs* 8.0 mo, hazard ratio (HR): 0.85,  $P = 0.048$ ] [40]. However, being a chimeric antibody, cetuximab may ultimately result in immunogenic responses.

To minimize the immunogenic responses, the fully humanized antibody panitumumab was developed. Panitumumab, unlike cetuximab, does not cause antibody-dependent cell-mediated cytotoxicity[41]. In the PRIME trial, the combination regimen of FOLFOX plus panitumumab outperformed FOLFOX alone in terms of PFS (10.0 mo *vs* 8.6 mo, HR: 0.80,  $P = 0.01$ ) and OS (23.9 mo *vs* 19.7 mo, HR: 0.88,  $P = 0.17$ ), with further evidence of significance in the updated survival analysis (HR: 0.83,  $P = 0.003$ ) in patients with mCRC[42,43].

Humanized antibodies are also a bit immunogenic compared to human antibodies. Necitumumab is a completely human monoclonal antibody approved by the FDA for advanced squamous non-small cell lung cancer. Necitumumab plus modified FOLFOX6 was studied in a phase II study for the first-line treatment of locally advanced or mCRC[44]. PFS and OS were 22.5 mo (11.0-30.0) and 10.0 mo (7.0-12.0),

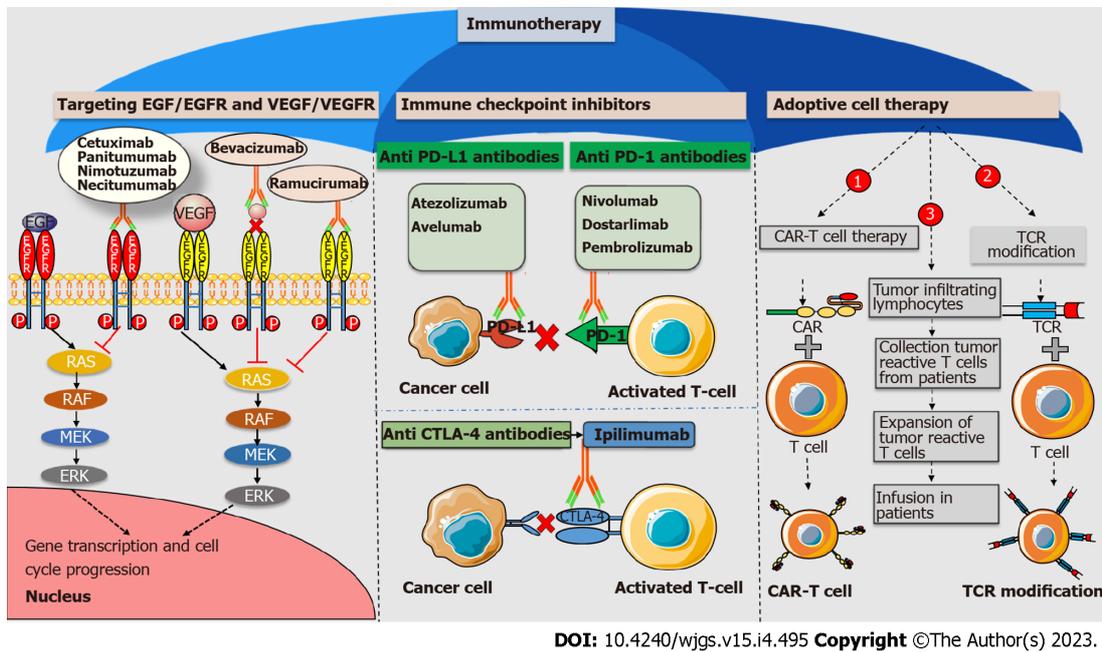
Table 1 Tyrosine kinase inhibitors, plant based drugs, and selected target specific agents against colorectal cancer

Agent	Type of agent	Target/mechanism	FDA approval date/trial number/status	Sources/interventions	Results
Sunitinib	TKI	VEGFR1-3	NCT00457691. Completed	Phase II study: FOLFIRI and sunitinib for mCRC	Sunitinib did not add to the antitumor activity of FOLFIRI
Axitinib	TKI	VEGFR1-3	NCT00460603. Completed	Phase II study: axitinib and/or bevacizumab with modified FOLFOX-6 as first-line therapy for mCRC	Neither the addition of continuous axitinib nor the axitinib/bevacizumab combination to FOLFOX-6 improved ORR, PFS, or OS compared with bevacizumab as first-line treatment of mCRC
Sorafenib	Kinase inhibitor	VEGFR	NCT00326495. Completed	Phase II study: cetuximab and sorafenib for the treatment of KRAS-mutated mCRC	No objective responses were observed
Regorafenib	Multikinase inhibitor	VEGFR1-3, TIE2, KIT, RET, RAF, PDGFR-B, FGFR	September 27, 2012	Approved for ACRC, mCRC	-
Encorafenib	Kinase inhibitor	BRAF-V600E as well as wildtype BRAF and CRAF	April 8, 2020	Approved for mCRC	-
Simtuzumab	Monoclonal antibody	LOXL2	NCT01479465. Completed	Phase II study: efficacy of simtuzumab with FOLFIRI as second line treatment in CRC	The addition of simtuzumab to FOLFIRI did not improve clinical outcomes in patients with metastatic KRAS-mutant CRC
Lenvatinib	TKI of VEGFR	VEGFR1-3, KIT, RET, PDGFR-alpha, FGFR	NCT04776148. Ongoing	Phase III study ongoing: lenvatinib in combination with pembrolizumab for mCRC	Ongoing
Tivozanib	TKI of VEGFR	VEGFR1-3	NCT01058655. Completed	Phase II study: everolimus (RAD001) and tivozanib (AV-951) in patients with refractory or mCRC	The oral combination of tivozanib and everolimus was well tolerated, with stable disease achieved in 50% of patients with refractory or mCRC
Tipifarnib	Farnesyltransferase inhibitor	Farnesyltransferase	NCT00005833. Completed	Phase II trial study: R-115777 given as a single agent	Ineffective in patients with mCRC
D-1553	Small molecule KRasG12C inhibitor	KRAS G12C	NCT04585035. Ongoing	Phase I study using D-1553 in CRC with KRAS G12C mutation	Ongoing
Aflibercept	Recombinant fusion protein	VEGF-A and VEGF-B, PGF	NCT02181556. Completed	Phase II study: aflibercept in combination with FOLFIRI as first-line chemotherapy in patients with mCRC	Although the primary objective was not met, first-line FOLFIRI + aflibercept for mCRC resulted in median PFS and OS close to those reported with traditional doublet and targeted therapies
Berberine	Alkaloid	Anti-proliferation, cell cycle arrest	<i>In vitro</i> study	Plant/berberine	Berberine inhibited telomerase activity and induced cell cycle arrest and telomere erosion in colorectal cancer cell Line, HCT 116[149]
<i>Piper nigrum</i> ethanolic extract	Alkaloid	Antioxidative activity	<i>In vitro</i> study	Plant/EEP	Time- and dose-dependent increase in the cytotoxic efficacy of 50% EEPN against colorectal carcinoma cell lines were noted[150]
Fucoidan	Polysaccharide	Inhibit growth and angiogenesis	<i>In vitro</i> study	Brown seaweed/combination of fucoidan with vitamin C	The combination of fucoidan with vitamin C showed significant inhibitory effects

Curcumin	Polyphenol	Apoptosis, antiangiogenesis, and cell cycle arrest	NCT02439385. Completed	Plant/phase II study: bevacizumab/FOLFIRI with ginsenoside-modifies nanostructured lipid carrier containing curcumin (G-NLC) in patients with mCRC	on HCT-116 colon cell viability[151]
			NCT01490996. Completed	Phase I/II study: curcumin combined with FOLFOX	Curcumin with FOLFOX was safe and tolerable. The HR for PFS and OS was 0.57 and 0.34, respectively
Gingerol	Polyphenol	Antioxidative and anti-inflammatory	NCT01344538. Completed	Plants/phase II randomized control trial. Ginger for CRC prevention	Result suggested ginger may reduce proliferation and increase apoptosis
EPA	Polyunsaturated fatty acids	Inhibit angiogenic factors	NCT00398333. Terminated	Marine microalgae/phase IV	Due to small sample size further investigation needed
EGCG	Polyphenol	Apoptosis	NCT02891538. Ongoing	Plants/early phase 1 study: EGCG in CRC patients	Ongoing
PSK	Polysaccharide	Apoptosis and antiproliferative	NCT00497107. NA	Fungi/phase III study: oral tegafur/uracil plus PSK	Results suggested that there was reduction in recurrence and mortality by 43.6% and 40.2%, respectively in stage I and stage II
Resveratrol	Polyphenol	Apoptosis and antiproliferative	NCT00920803. Completed	Plants/phase I study: resveratrol for resectable CRC	Resveratrol was effective in treating CRC by modulating the Wnt pathway
Topotecan	Alkaloid	Antiproliferative	EORTC	Plants/phase II study: oral topotecan	Topotecan administered as a five times daily regimen has only minor activity as a single-agent therapy in colorectal cancer
Metformin	Alkaloid	Antiproliferative and antimetastatic	NCT03047837. NA	Plants/phase II study: using aspirin and metformin in stage I-II CRC	Result suggested that the given intervention delayed recurrence and improved prognosis
Everolimus	Macrolide	Antiproliferative and antimetastatic	NCT01387880. Completed	Bacterial/phase II study: irinotecan, cetuximab, and everolimus to patients with mCRC	Everolimus showed promising effects on CRC prognosis
			NCT01058655. Completed	Phase II study: tivozanib and everolimus for patients with refractory mCRC	Oral combination of tivozanib and everolimus was well tolerated in 50% of the patient
Andrographolide	Diterpenoid	Apoptosis, antiproliferative, and cell cycle arrest	<i>In vitro</i> study	<i>In vitro</i> study using 5-FU with andrographolide	Andrographolide enhanced 5-FU induced antitumor effect in CRC <i>via</i> inhibition of the c-MET pathway[152]
Silymarin	Flavonoid	Apoptosis, antiproliferative	NCT03130634. Completed	Plants/phase IV study using silymarin in patients treated with first-line treatment FOLFIRI	Silymarin is a potential supplement for reducing toxicities in mCRC patients undergoing FOLFIRI plus bevacizumab first-line treatment
MMC	Hyleneimines	Antiproliferative	NCT00643877. NA	Streptomyces/phase III study using PHARC with oxaliplatin, MMC FUDR	Addition of PHARC improved DFS in patients with stage II and stage III CRC
			NCT03073694. Ongoing	Phase II study using MMC and melphalan	Ongoing

5-FU: 5-fluorouracil; ACRC: Advanced colorectal cancer; *BRAF-V600E*: *BRAF* protein coding gene; CRC: Colorectal cancer; DFS: Disease-free survival; EEPN: Ethanolic extract of *Piper nigrum*; EGCG: Epigallocatechin gallate; EORTC: European Organization for Research and Treatment of Cancer; EPA: Eicosapentaenoic acid; FDA: Food and Drug Administration; FGFR: Fibroblast growth factor receptor; FOLFIRI: 5-fluorouracil, leucovorin, and irinotecan; FOLFOX-6: 5-fluorouracil, leucovorin, and oxaliplatin-6; FUDR: Fluorodeoxyuridine; G-NLC: Genistein-nanostructured lipid carriers; HR: Hazard ratio; KIT: Proto-oncogene receptor tyrosine kinase; LOXL2: Lysyl oxidase-like 2; mCRC: Metastatic colorectal cancer; MMC: Mitomycin C; NA: Not available;

ORR: Objective response rate; OS: Overall survival; PDGFR-B: Platelet-derived growth factor receptor B; PFS: Progression-free survival; PGF: Placental growth factor; PHARC: Preoperative hepatic and regional atrial chemotherapy; PSK: Polysaccharide krestin; RAF: Rapidly activated fibrosarcoma; RET: Ret proto-oncogene; TIE2: Tyrosine kinase with immunoglobulin and epidermal growth factor 2; TKI: Tyrosine kinase inhibitor; VEGFR1-3: Vascular endothelial growth factor 1-3.



**Figure 1 Immunotherapeutic approaches against colorectal cancer.** Antiangiogenic monoclonal antibodies like cetuximab, panitumumab, nimotuzumab, and nectinumab target epidermal growth factor (EGF) receptor (EGFR). Antiangiogenic monoclonal antibodies such as bevacizumab and ramucirumab target vascular EGF and its receptor, respectively. All antiangiogenic monoclonal antibodies downregulate the RAS-RAF-MEK-ERK pathway and prevent the transcription of genes involved in cell cycle progression. Immune checkpoint inhibitors (ICIs) like atezolizumab and avelumab target programmed cell death ligand 1 (PD-L1). ICIs like nivolumab, dostarlimab, and pembrolizumab target programmed cell death protein 1 (PD-1). The ICI ipilimumab targets cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). ICIs act as immune brakes that prevent checkpoint proteins from interacting with their companion proteins, thus boosting T cell effector activity. T cell boosting therapy, like adoptive cell therapy, includes chimeric antigen receptor T cell therapy (CAR-T), T cell receptor modification (TCR), and tumor infiltrating lymphocyte boost T cell activity to combat cancer cell growth. VEGF: Vascular epidermal growth factor; VEGFR: Vascular epidermal growth factor receptor.

respectively. First-line nectinumab modified FOLFOX6 effectively treated locally advanced or mCRC with manageable side effects; further research on the effect of nectinumab on RAS-associated mutation is necessary. Humanized anti-EGFR antibodies, such as nimotuzumab, have a better safety record than most anti-EGFR antibodies.

In clinical practice, there were no discernible differences in the objective RR, disease control rate, PFS, or median survival between chemotherapy plus nimotuzumab and chemotherapy alone in patients with advanced CRC, indicating that these treatments were equally effective for treating the disease[45]. Both cetuximab and panitumumab are FDA-approved first-line treatments for CRC. Anti-EGFR medicines may be a low priority for second-line or beyond CRC treatment because cetuximab and panitumumab failed to achieve statistically superior PFS or OS for patients with CRC in multiple studies[46,47].

**Anti-VEGF/VEGFR antibodies:** Angiogenesis is required for tumor invasion and metastasis to progress beyond the size of a few centimeters. In CRC and other malignancies, VEGF is the most important angiogenic factor. As a result, multiple studies examining VEGF expression in CRC patients have been conducted, and several therapeutic drugs targeting the VEGF pathway have been tested. Bevacizumab, an antiangiogenic antibody, has proven to be more effective than chemotherapy when combined with IRI, 5-FU, and LV plus placebo in phase II and III AVF2107 studies based on antiangiogenic therapy for CRC. The AVF2107 study found that bevacizumab, a humanized IgG monoclonal antibody, targets VEGF-A and improves both PFS and OS in mCRC (RR: 44.0% vs 34.8%; OS: 20.3 mo vs 15.6 mo; HR: 0.66,  $P = 0.001$ ; PFS: 10.6 mo vs 6.2 mo; HR: 0.54,  $P = 0.001$ ).

In the E3200 trial, patients with CRC who progressed after FOLFOX therapy had a better PFS (7.3 mo vs 4.7 mo, HR: 0.61,  $P = 0.001$ ) and OS (12.9 mo vs 10.8 mo, HR: 0.75,  $P = 0.0011$ ) as well as a better RR (22.7% vs 8.6%,  $P = 0.0001$ ) with a combination of FOLFOX and bevacizumab than FOLFOX alone. Bevacizumab is the only antibody approved by the FDA as a first- and second-line VEGF-targeted therapy for CRC. Another FDA-approved medication for second-line therapy of mCRC is ramucirumab, a completely humanized monoclonal VEGFR-2-targeted IgG antibody. Compared to FOLFIRI-placebo, a

combination of ramucirumab and FOLFIRI significantly improved PFS (5.7 mo *vs* 4.5 mo; HR: 0.79,  $P = 0.0005$ ) and OS (13.3 mo *vs* 11.7 mo, HR: 0.84,  $P = 0.022$ ) based on the phase III RAISE trial. Table 2 enlists various anti-VEGF/VEGFR and anti-epidermal growth factor (EGF)/EGFR monoclonal antibodies that have been approved and are undergoing preclinical and clinical trials. All these medications increased OS from a few weeks to months. However, some tumor characteristics, such as genetic changes in endothelial cells, vasculogenic mimicry, and the unique therapeutic response of each tumor, interfere with these antiangiogenic therapeutic approaches. Furthermore, angiogenic treatments promote a hypoxic environment, which promotes tumor invasiveness[48].

**ICIs as immune boosters:** The most common immunomodulatory antibodies are ICIs. ICIs are immune brakes that prevent checkpoint proteins from interacting with their companion proteins. The important immune checkpoints and their immunological inhibitors are listed in Table 2. One important target protein is cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). CTLA-4 controls T cell activation and transmits inhibitory signals to T cells[49]. CTLA-4 inhibition by the anti-CTLA-4 antibody was shown to inhibit tumor progression by upregulating effector T cell activity and suppressing regulatory T cells [50]. The FDA has approved low-dose ipilimumab (CTLA-4 inhibitor) in combination with nivolumab for previously treated microsatellite instability-high and deficient mismatch repair (MSI-H/dMMR) mCRC[51].

Programmed cell death receptor programmed cell death protein 1 (PD-1) is another ICI target; it is a type 1 transmembrane protein that interacts with programmed cell death ligand 1 (PD-L1) (highly expressed in inflamed cells) and PD-L2 (expressed only on antigen-presenting cells). The interaction of PD-1 and PD-L1/PD-L2 is immunosuppressive, limiting the function of T effector cells to minimize the over storm of immune responses[52,53]. However, in CRC, overexpression of PD-1 and PD-L1/PD-L2 causes more than required immunosuppression[54]. Monoclonal antibodies were developed to bind and prevent PD-1/PD-L1 interactions and to assist T cells in killing cancerous cells by restoring their activation, proliferation, function, and downstream immune signaling[55]. There are five FDA-approved PD-1 inhibitors; three are approved for CRC (Table 2).

Pembrolizumab and nivolumab (PD-1 inhibitors) are used in CRC. Pembrolizumab improves CRC patients with dMMR/MSI-H based on the KEYNOTE-177 trial. Results showed a RR of up to 78% compared to 11% of patients with proficient mismatch-repair and microsatellite stability CRC[56]. Another successful PD-1 inhibitor is nivolumab, which shows durable responses with a 69% OS rate of 12 mo among patients with the dMMR mCRC[51]. Further, a higher RR of up to 94% in MSI-H/dMMR mCRC has been observed in the phase II CheckMate study (NCT02060188), which used the combination of nivolumab (a PD-1 targeting antibody) and ipilimumab (a CTLA-4-targeting antibody)[57]. This implies that the combination of immune checkpoint therapy can significantly increase the effectiveness of the treatment for MSI-H/dMMR mCRC patients.

Other potential immune checkpoints are lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin, and mucin domain-3-containing molecule 3 (TIM-3). They play an important role in T cell regulation and preventing autoimmune disorders. However, in CRC, LAG-3[58] and TIM-3[59] are overexpressed in the CRC microenvironment, which diminishes the effectiveness of T effector cells. Relatlimab is a monoclonal antibody that binds to LAG-3 and restores T cell effectiveness. Numerous clinical trials are currently being conducted on it (NCT03642067 and NCT05328908). IBI104 is a brand-new, highly-promising antibody that inhibits TIM-3-mediated suppressive signaling and as a result is crucial for the regulation of T cells. No research on the role of IBI104 in CRC has been conducted, and it can be added to the list of new explorable T cell boosters in CRC.

### **T-cell boosting therapies**

**Adoptive cell transfer therapy:** Adoptive cell transfer therapy (ACT) is a cell-based therapy that uses cells from the patient (autologous transfer) or from other donors (allogeneic transfer) to improve immune function[60]. The ACT is performed in three ways: using tumor-infiltrating lymphocytes (TILs), inserting CAR, and modifying TCR. TILs are produced from the tumor-reactive T cells from the patients, expanded *ex vivo*, and infused in the patients. TILs are capable of identifying several targets in cancer cells. Numerous active clinical trials are investigating the potential of TILs to treat CRC (NCT01174121, NCT03935893, and NCT03610490). However, a significant shortcoming of ACT-TIL is the inability to produce tumor-specific T cells when employing patients' own TIL.

Another ACT is CAR-T cells, in which T cells are derived from a patient or donor and fused with variable antibody fragments specific to the target antigen[61]. T cells are taken from a patient (autologous) or human leucocyte antigen-matched donor (allogeneic), cultivated *ex vivo*, and genetically transformed as CAR-T cells by inserting the CAR onto the T cells in the CAR approach. The main target of CAR-T cells are carcinoembryonic antigens (CEA), guanylyl cyclase C[62,63], tumor-associated glycoprotein 72[64], epithelial cell adhesion molecule[65], and major histocompatibility complex class I-related chain A and B. A clinical trial is ongoing targeting CEA to treat CRC liver metastases (NCT05240950).

Another newly developed ACT strategy includes altering TCR. Although it appears similar to CAR-T cells, its antigen-recognition processes are different. ACT, like many other approaches, has many drawbacks. Due to the necessity to create tumor-specific lymphocytes for each patient, this strategy is

Table 2 Immunotherapeutic agents and vaccines against colorectal cancer

Antibodies/antigenic composition	Origin	Target/CRC stage	Approval date/trial number/yr	Description/interventions	Inference
Monoclonal antibodies					
Cetuximab	Chimeric	EGFR	February 12, 2004	Cetuximab alone for mCRC	Adding cetuximab to first-line chemotherapy in patients with WT <i>KRAS</i> mCRC was statistically beneficial for OS and PFS[153]
			July 6, 2012	For mCRC cetuximab + FOLFIRI	
Panitumumab	Humanized	EGFR	September 27, 2006	For mCRC panitumumab + FOLFOX for WT <i>KRAS</i> mCRC. For WT <i>RAS</i> mutation mCRC	In WT <i>KRAS</i> mCRC, PFS was improved, objective response was higher, and there was a trend toward improved OS with panitumumab-FOLFOX4[42,43,154]
			May 23, 2014		
			June 29, 2017		
Nimotuzumab	Humanized	EGFR	NCT05278728. Completed	Phase II study nimotuzumab along with radiotherapy and concurrent capecitabine	No significant outcomes
			NCT05278728. Completed	Phase IIa study of nimotuzumab to treat CRC	Ongoing
Necitumumab	Human	Cetuximab-resistant EGFR	NCT00835185. Completed	Phase II study necitumumab plus modified FOLFOX6 for locally advanced and mCRC	First-line necitumumab + mFOLFOX6 was active with manageable toxicity in locally advanced or mCRC
Bevacizumab	Humanized	VEGF	February 26, 2004	For mCRC	The addition of bevacizumab to 5-fluorouracil-based combination significantly increased patient survival[155,156]
Ramucirumab	Human	VEGFR-2	April 24, 2015	Ramucirumab with FOLFIRI as second-line treatment for mCRC	The addition of ramucirumab to FOLFIRI improved patient outcomes in the RAISE trial[157]
Nivolumab	Human	PD-1	August 1, 2017	Nivolumab approved for MSI-H/dMMR mCRC	Nivolumab provided durable responses and disease control in pre-treated patients with dMMR/MSI-H mCRC [51]
Ipilimumab	Human	CTLA-4	July 11, 2018	Nivolumab plus low dose ipilimumab approved for previously treated MSI-H/dMMR mCRC	Clinical effect with nivolumab plus low-dose ipilimumab was significant and long-lasting for MSI-H/dMMR mCRC[57]
Cemiplimab	Human	PD-1	NCT04157985. Ongoing	Phase III study: evaluating length of treatment with cemiplimab and other inhibitors in solid tumor patients	Ongoing
Atezolizumab	Humanized	PD-L1	NCT02788279. Completed	Phase III study: atezolizumab with or without cobimetinib <i>vs</i> regorafenib in previously treated mCRC	Did not meet its primary endpoint of improved OS with atezolizumab plus cobimetinib or atezolizumab <i>vs</i> regorafenib
			NCT05118724. Ongoing	Phase II study: atezolizumab with/without IMM-101 in patients with MSI-H/dMMR stage III CRC ineligible for oxaliplatin	Ongoing
			NCT05456165. Ongoing	Phase II study: atezolizumab in combination with neoantigen	Ongoing

Avelumab	Human	PD-L1	NCT03854799. Ongoing	targeting vaccine Phase II study: avelumab + capecitabine combined with radiation	Ongoing
Dostarlimab	Humanized	PD-1	NCT03475953. Ongoing	Phase I/II Study: regorafenib plus avelumab in solid tumors	Ongoing
Dostarlimab	Humanized	PD-1	NCT04165772. Ongoing	PD-1 blockade in dMMR, locally advanced rectal cancer	Ongoing; dMMR, locally advanced rectal cancer was highly sensitive to single-agent PD-1 blockade. Longer follow-up is needed to assess the duration of response
Pembrolizumab	Humanized	PD-1	June 29, 2020	Pembrolizumab for first-Line treatment of patients with unresectable or metastatic MSI-H or dMMR CRC	Approved based on Phase III Keynote-117 Trial in which pembrolizumab significantly reduced the risk of disease progression or death by 40% [158]
Relatimab	Human	LAG-3	NCT05328908. Ongoing	Phase III study of nivolumab-relatlimab fixed-dose combination <i>vs</i> regorafenib or TAS-102 in participants with mCRC	Ongoing
Relatimab	Human	LAG-3	NCT03642067. Ongoing	Study of nivolumab and relatlimab in patients with MSS advanced CRC	Ongoing
Peptide based vaccines[80]					
SART3	-	Metastatic	2001	Used with adjuvant incomplete Freund's adjuvant	Increased cellular immune responses to both CRC cells and the vaccinated peptide
Recombinant Ep-CAM (with liposome carrier)	-	I-III	2001	Used with adjuvant alum	The overall immune response was safe and effective for patients with CRC and advanced cancer against Ep-CAM
		II-IV	2004	Used with adjuvant GM-CSF	
		Metastatic	2004	Used with adjuvant GM-CSF	
CIP37-DT	-	III-IV	2002	Used with adjuvant Nor-MDP (Muramyl dipeptide)	Longer OS with an excellent safety profile in patients with CRC
Recombinant CEA expressed in baculovirus system	Expressed in baculovirus-insect cell system	Stage I-III	2004	Used with adjuvant alum and GM-CSF	Potent and long lasting antigen specific IgG and T cell response
Survivin-2B Human	-	Metastatic	2004	Used with adjuvant UFT (uracil-tegafur)	Excellent safety profile with potent immune response against HLA-A24-expression in patients with CRC
G17DT (N-terminus of gastrin 17)	-	Metastatic	2014	Used with adjuvant diphtheria toxoid	In combination with irinotecan this vaccine has an acceptable immune response with significantly longer survival
OCV-C02	-	Metastatic	2017	Two peptide epitopes derived from RNF43 and TOMM34 and used with adjuvant montanide ISA 51	Safe immune response in recurrent or advanced stage CRC patients resistant to standard chemotherapy
RNF43 and TOMM34-derived peptides	-	III	2018	Used with uracil-tegafur/leucovorin, montanide ISA 51	Strong immune response with increased OS in patients with stage III CRC
PolyPEP11018	-	Metastatic	2020	Used with adjuvant montanide ISA 51 Human	Safe and well-tolerated and induced robust CRC-specific T cell responses, similar to

					personalized neoantigen vaccines
mRNA-based vaccines[80]					
NCI 4650 (mRNA 4650)	-	Metastatic	2019	-	Partly safe and neoantigen specific CD8 and CD4 T cells responses against CRC neoepitopes
mRNA 4157	-	Metastatic	2019	In combination with pembrolizumab	Partly safe and strong neoantigen specific T cell responses against CRC neoepitopes
V 941 (mRNA 5671)	-	Metastatic	2019, NCT03948763	In combination with pembrolizumab	KRAS vaccine clinical trial is underway, and the results are eagerly awaited
RO 7198457 (RG 6180)	-	Metastatic	2020	In combination with atezolizumab	Partly safe and strong neoantigen specific immune responses
Cell based vaccines[80]					
Tumor cell	Tumor cell	II and III	2000	In combination with BCG	Less potency with 5-yr OS of 84.6%
Cancer Vax	Tumor cell	IV	2001	In combination with BCG	Significant increase in anti-TA90 IgG and IgM titers, and the OS was 21.9 mo
HSPPC-gp96	Tumor cell	IV	2003	-	Two-year overall survival and disease-free survival improved
CEA mRNA	DCs	IV	2003	In combination with IL-2	Well tolerated and safe immunization observed in patients with advanced malignancies
OPA-DC	DCs	Metastatic	2011	CEA peptide-loaded DCs matured with a combination of OK432, prostanoid, and interferon- $\alpha$	Increased CEA-specific cytotoxic T cell response and NK cell levels in 8 patients with stable disease
Autologous tumor lysate DC (ADC)	DCs	Metastatic	2016	-	Not recommended: the use of ADC alone, in a phase III trial
Autologous tumor antigens-loaded DC	DCs	Metastatic	2018	In combination with 5-fluorouracil	Treatment was safe and had shown particularly prominent IL-12 production for immunization against neoantigens
Vector based vaccines					
ALVAC-CEA/B7	Canary pox virus vector	Metastatic	2008; 2013	In combination with chemotherapy	Acceptable safety profile and induced CEA-specific T cell responses in patients with mCRC
AVX701	Alphavirus vector	III	2010	VRP expressing CEA	Well tolerated and elicit robust CEA-specific T cell and antibody responses in patients with CRC
GI-6207	<i>Saccharomyces cerevisiae</i>	Metastatic	2014	-	Strong antigen-specific CD8+ T cells and CD4+ T responses and extended stable disease
GI-6301	<i>Saccharomyces cerevisiae</i>	Metastatic	2015	-	Decreased tumor density and serum CEA levels in CRC treated patients

pLADD	<i>Listeria monocytogenes</i>	Metastatic	2017, NCT03189030	<i>Listeria</i> bacterial vector in combination with neoantigens	Induced neoantigen-specific CD8+ T cells and gamma delta T cells
Cholera	Bacteria	I-IV	2018	-	Cholera vaccination largely decreased the mortality rate of CRC
GI-4000	<i>Saccharomyces cerevisiae</i>	Metastatic	2018	-	Excellent safety profile and favorable immunogenicity in the majority of subjects
ADXS-NEO	-	Metastatic	2019	Bacteria expressing personalized tumor antigens	Increased CD4+/CD8+ T cell-mediated immune response

ADC: Antibody drug conjugate; BCG: Bacillus Calmette–Guérin; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; CTLA-4: Cytotoxic T-Lymphocyte-associated antigen-4; DCs: Dendritic cells; dMMR/MSI-H: Microsatellite instability high and deficient mismatch repair; EGFR: Epidermal growth factor receptor; Ep-CAM: Epithelial cell adhesion molecule; FOLFIRI: 5-fluorouracil, leucovorin, and irinotecan; FOLFOX4/6: 5-fluorouracil, leucovorin, and oxaliplatin; GM-CSF: Granulocyte macrophage colony-stimulating factor; HLA: Human leukocyte antigen; HSPPC-gp96: Heat shock protein peptide glycoprotein Complex-96; Ig: Immunoglobulin; IL: Interleukin; LAG-3: Lymphocyte activation gene 3; mCRC: Metastatic colorectal cancer; MSS: Microsatellite stable; NK: Natural killer cell; Nor-MDP: Nor-muramyl dipeptide; OS Overall survival; PFS: Progression-free survival; PD-1: Programmed cell death receptor 1; PD-L1: Programmed cell death ligand 1; RNF43: Ring finger protein 43; SART3: Squamous cell carcinoma antigen identified by T cells 3; TOMM34: Translocase of outer mitochondrial membrane 34; VEGF: Vascular endothelial growth factor; VEGFR: Vascular endothelial growth factor receptor; WT: Wild-type.

technically and economically demanding for both the business and patients. Second, the allogeneic transplant patient is frequently at risk of graft-versus-host disease. Although ACT offers considerable therapeutic promise, several issues still need to be carefully considered.

**Vaccines:** Cancer vaccines elicit an immune response by exposing tumor-associated antigens (whether in free form or expressed on the cell surface) to the immune system, leading to immune cell-mediated cancer cell death. Several clinical trials have been conducted over the past two decades introducing novel vaccines against CRC and other carcinomas[66]. Several possible tumor-associated antigens have been targeted over the past decades against CRC, such as squamous cell carcinoma antigen identified by T cells 3[67], survivin[68], CEA[69], melanoma-associated antigen[70], translocase of outer mitochondrial membrane 34[71], insulin-like growth factor-II mRNA binding protein 3[72], EGFR[73], transmembrane 4 superfamily member 5 protein[74], VEGFR1, Wilms tumor 1 protein[75], and ring finger protein 43[76]. These vaccines activate local immune cells by releasing tumor antigens and increase immune cell, like T cells and dendritic cells, infiltration to the site of action[77].

Several vaccination strategies have been developed against cancers based on antigenic composition. Depending upon the molecular composition of the antigen, vaccine methods can be classified as molecular-based, cell-based, and vector-based. Molecular-based vaccines consist of full-length peptides and DNA and mRNA vaccines. The CRC clinical trials showed that the mRNA and DNA vaccines have demonstrated impressive antitumor response with remarkable efficacy and safety profiles compared to the peptide vaccines. Cell-based vaccines consist of native and genetically modified tumor cells and activated dendritic cell vaccines. These vaccine strategies have reported limited efficacy in several trials but have shown potential efficacy in combination with chemotherapy and immunotherapy[78,79].

Live attenuated viruses, yeasts, and bacterial vectors are vector-based vaccines. The oncolytic viral vectors are used for the live attenuation virus vaccine strategy; however, due to increased immunogenicity, the clinical efficacy remains limited. The live attenuated bacterial and yeast vector-based vaccine are the emerging option for therapeutic vaccines against CRC[80]. These different type of vaccines discussed above have been evaluated in several clinical trials in the past decade, listed in Table 2.

**Role of cytokines in CRC:** Various cells, primarily immune cells (T cells, neutrophils, and macrophages), endothelial cells, fibroblasts, and other stromal cells, secrete cytokines[81]. Due to the complex network of cytokines in immune responses, cytokine-based medications are a complex task requiring a thorough understanding of cytokine biology and modern biotechnology to maximize antitumor activity while minimizing toxicity. Many cytokines therapy trials are currently ongoing for various cancer[82], but for CRC, there is a need for preclinical studies to assess their unexpected toxicity. From a future perspective, cytokines can be essential molecules because of their capacity to increase and reactivate natural killer (NK) cells and T lymphocytes, encourage lymphocyte infiltration of tumors, and persist in the tumor microenvironment (TME).

Despite the fact that immunotherapy is a cutting-edge and intriguing cancer treatment method, it comes with long-term effects and shortcomings. The main limitation of monoclonal antibodies is the decrease in efficacy because of the short half-life[83]. Immune checkpoints defend the body against infections and autoimmune diseases to maintain immune system equilibrium. ICIs induce the activation

of T lymphocytes. However, sustained overactivation of these cells causes immune-related side effects including systemic toxicity[84]. ICIs do not elicit a response in all patients as their T cells are not potent enough to recognize malignant cells. Immunotherapy that boosts T cells does not work against solid tumor cells. Immunosuppressive cells that release chemokines like CXCL1, CXCL5, and others are prevalent in solid tumor cells, and they also have a lot of fibrous matrix[85,86].

CAR-T cells are not very effective in infiltration of solid tumor cells because of their architecture[87]. For each patient using the ACT approach, tumor-specific lymphocytes had to be produced. As a result, it is difficult financially and technically for both the industry and the patients. In the long run, the patients are at a significant risk of developing cytotoxicity, such as B cell hyperplasia, cytokine release syndrome, and graft-versus-host disease[83].

In cases of vaccination, several trials have reported limited efficacy. This is due to the rejection upon identification as a foreign material[88]. In cytokine base therapy, increased levels of cytokines like interleukin-6 and interferon- $\gamma$  in the bloodstream cause life-threatening poisoning. High toxicity and excessive immune response are caused by immunological signaling and immune cell stimulation brought on by an excess of cytokines[89]. When cytokines are used in excess for an extended period of time, neurological issues like hallucinations, comas, seizures, and verbal difficulties can also arise. Cytotoxicity and a cytokine storm are caused by them[83].

### Other approaches

**Non-coding RNA-mediated therapy:** According to high-throughput genome-scale research, 5%-10% of the transcribed sequences are translated into mRNA or non-coding RNA (ncRNA), out of which only 1% is composed of protein-coding genes and the remaining 4%-9% is transcribed into ncRNAs. As a result, ncRNAs make up a sizable fraction of all RNA molecules[90]. Short regulatory ncRNAs, such as miRNAs and siRNAs, were first identified for their function and clinical significance. The regulatory functions of miRNAs in nearly all physiological and pathological processes in the body, including carcinogenesis, have been widely acknowledged. Several miRNAs are dysregulated in plasma samples of CRC patients; therefore, they have good diagnostic value for CRC screening[90].

Apart from tumor diagnosis and prognosis, ncRNAs can be interesting therapeutic targets for CRC given their tumor suppressive and oncogenic properties. Some ncRNAs play a crucial role in tumorigenesis by losing their tumor suppressive activity, and some are aberrantly overexpressed in CRC, contributing to the oncogenic activity. Therefore, these two mechanisms are used for the development of ncRNA-oriented therapies[91]. Over the past two decades, several synthetic miRNAs and siRNAs have been administered to animal models of various diseases. However, the proper delivery of synthetic miRNAs and siRNAs at the required site of action remains questionable. Several difficulties, including the inability of negatively charged genes to enter negatively charged cellular membranes, gene destruction by plasma nucleases, non-specificity towards targeted cells, and other issues, make *in vivo* direct administration of naked therapeutic genes impractical and challenging. Therefore several drug delivery strategies such as gene transfection with the help of viral or non-viral vectors, nanodelivery system, and others are safe and effective for delivery of siRNAs and miRNAs to the site of action[92].

Ever since the discovery of the potential role of miRNAs as therapeutic targets, several studies have been conducted aimed at various mechanisms such as miRNA-mediated chemosensitization and miRNA mimicking for tumor suppression and oncogenic miRNA targeting[93]. miRNA/siRNA combinatorial therapy with various chemotherapeutic and immunotherapeutic agents has increased the sensitivity of these drugs to cancers[94]. However, most of the studies performed on CRC are limited to animal models and CRC cell lines, and none were extrapolated to CRC patients[95]. Despite the advances in research, no miRNA or siRNA therapy are FDA approved for clinical usage against CRC or any other cancer, but several are in clinical trials (Table 3).

**Oncolytic viral therapy:** Oncolytic viral therapy or immuno-oncolytic virotherapy employs native or sometimes genetically modified viruses called oncolytic viruses to predominantly infect cancerous cells while avoiding healthy ones[96]. It has emerged as a promising treatment option for CRC. Oncolytic viruses are thought to exert antitumor activity *via* two distinct mechanisms: induction of systemic antitumor immunity and selective replication within neoplastic cells that have a direct lytic effect on the tumor cells. The specificity of oncolytic viruses for tumors can be increased by selective genetic modifications such as the expression of viral surface proteins that bind to cellular receptors found only on cancerous cells, deletion of virus fatal genes, and the addition of different immunostimulatory genes [97].

In past decades, several oncolytic viruses have been evaluated experimentally against CRC, including adenovirus, vaccinia virus, herpes simplex virus, vesicular stomatitis virus, measles virus, tanapox virus, echovirus, reovirus, and Newcastle disease virus. Several oncolytic viruses were also tested clinically in CRC patients as monotherapy, and combinatorial therapy of oncolytic viruses with chemotherapeutics[98], radiotherapy[99], and immunotherapeutics have also been examined against CRC[100]. In the first phase I/II trial, patients with refractory CRC treated with herpes simplex mutant NV120 had good safety profiles and increased chemosensitization[101]. A phase I clinical trial of an oncolytic Western Reserve strain genetically modified with deletion of vaccinia growth factor and thymidine kinase (vaccinia virus mutant) had shown an excellent safety profile in 17 patients with

**Table 3 microRNAs and small interfering RNAs as therapeutics for colorectal cancer in clinical trials**

Therapeutic name	Target gene/protein	Route of administration	Phase/status	Clinical trial identifier	Outcome
siRNA targeted therapeutics					
ALN-VSP02	<i>VEGF, KSP</i>	Systemic	Phase I (2011)/terminated	NCT00882180	It was well-tolerated and had antitumor activity
		IV infusion	Phase I (2012)/completed	NCT01158079	
Atu027	<i>PKN3</i>	Systemic	Phase I (2012)	NCT00938574	It was safe in patients with advanced solid tumors
		IV infusion	Phase I/II (2016)/completed	NCT01808638	
CALAA-01	<i>RRM 2</i>	Systemic; IV infusion	Phase I (2013)/terminated	NCT00689065	It was well tolerated during the initial dose escalation portion of the phase Ia study
siRNA-EphA2-DOPC	<i>EphA2</i>	Systemic; IV infusion	Phase I 2015/active	NCT01591356	It was well tolerated at all doses tested in preclinical studies
TKM-PLK1 (TKM-080301)	<i>PLK-1</i>	Systemic; IV infusion	Phase I/II (2016)/completed	NCT02191878	It was tolerated and showed preliminary antitumor efficacy
DCR-MYC (DCRM1711)	<i>MYC</i>	Systemic	Phase I (2017)	NCT02110563	It was well tolerated and showed promising initial clinical and metabolic responses across various dose levels
		IV infusion	Phase Ib/2 (2016)/terminated	NCT02314052	
NBF-006	<i>GSTP</i>	Systemic; IV infusion	Phase1 (2019)/active	NCT03819387	Significant tumor growth inhibition and overall survival benefit was observed
miRNA targeted therapeutics					
MRX34	miR-34a mimic	IV infusion	Phase I/terminated-2016. Phase I-II/withdrawn-2016	NCT01829971 NCT02862145	Unexpected severe immune-mediated toxicities observed

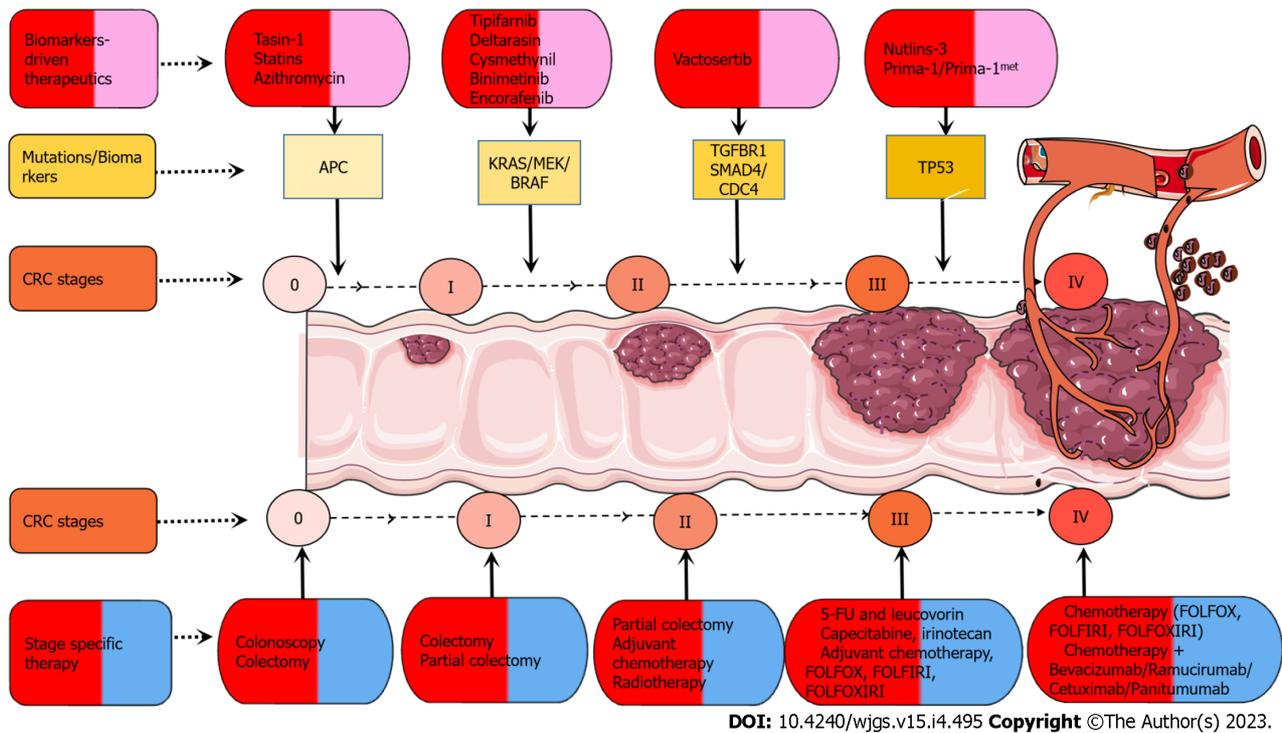
DCR-MYC: Lipid nanoparticle-formulated small inhibitory RNA; EphA2: Ephrin type-A receptor 2; GSTP: Glutathione S-transferase pi gene; IV: Intravenous; KSP: Kinesin spindle protein; miRNA: MicroRNA; PKN3: Protein kinase N3; PLK-1: Polo-like kinase 1; RRM2: Ribonucleotide reductase regulatory subunit M2; siRNA: Small interfering RNA; VEGF: Vascular endothelial growth factor.

advanced and refractory CRC. However, only 1 patient experienced a true benefit from the therapy [102]. This therapy was intratumoral, and another trial with the same agent, when given intravenously in 11 patients, had established safety but limited antitumor activity [103].

A phase II clinical trial of FOLFOX/bevacizumab with or without an oncolytic reovirus pelareorep in 103 patients with mCRC showed a significantly improved response (combined therapy); however, the OS was poor [104]. In another case, patients with stage IV CRC treated with a combined regimen of oncolytic enterovirus, FOLFOX/bevacizumab, and surgical resection (RIGVIR) had impressive efficacy with complete remission for 7.7 years after diagnosis [105]. Despite the intensive research in this field, none of the oncolytic viral therapy is FDA approved for routine clinical use against CRC. Challenges like evading host antiviral immunity and the successful delivery of the virus to the target site cause a hindrance to achieve the optimal antitumor activity of oncolytic viruses. However, several promising strategies like using stem cells or immune cells as carriers are in the pipeline and may increase the antitumor activity of oncolytic viruses.

**Cancer stage specific therapy:** The cancer stage determines how it will be treated, as given in Figure 2, although other aspects may also be significant. Stage 0 CRC does not spread past the inner lining of the colon; therefore, a colonoscope can be used most of the time to remove the polyp. A partial colectomy can be performed if the cancer is too large to be eliminated by local excision [106]. Many cancers that were a remnant of a polyp are included in stage I and have penetrated further into the layers of the colon wall. They have not migrated to surrounding lymph nodes or outside of the colon wall itself. The complete removal of the cancerous polyp may not require further treatment; however, if the polyp is high-grade, more surgery may be recommended. If the cancer is not generated from the polyp, partial colectomy is the standard treatment [107].

Stage II CRC has penetrated the wall of the colon and adjacent tissue but has not reached the lymph nodes. These require partial colectomy followed by adjuvant chemotherapy and/or radiotherapy to reduce the risk of relapse. However, adjuvant chemotherapy is not always used for stage II cancers due to severe adverse effects. Stage III CRC has penetrated the adjacent lymph nodes but has not migrated to the other body parts. The partial colectomy and adjoining lymph nodes followed by chemotherapy (FOLFOX or CAPOX) is the standard treatment regimen for this stage of CRC.



**Figure 2 Biomarker-driven therapeutics and cancer stage specific therapeutic strategies.** Drugs like tasin-1, statins, and azithromycin specifically target adenomatous polyposis coli (APC). Drugs such as tipifarnib, deltarasin, and cysmethynil target *KRAS* mutations in colorectal cancer (CRC), and binimetinib and encorafenib target *BRAF*-V600 mutations in CRC. Vactosertib is a potent and selective transforming growth factor-beta receptor I kinase inhibitor. Small molecule nutlins-3 is an MDM2 antagonist blocking the interaction between MDM2 and p53. Small molecules prima-1/prima-1met convert mutant p53 to an active conformation. There are stage-specific therapies for different stages. For stage 0, colonoscopy and colectomy are recommended. For stage I, colectomy and partial colectomy are recommended. For stage II, partial colectomy, adjuvant chemotherapy, and radiotherapy are recommended. For stage III, chemotherapy includes 5-fluorouracil (5-FU), 5-FU/leucovorin, capecitabine, irinotecan, adjuvant chemotherapy, and combinational chemotherapy [5-FU + leucovorin + oxaliplatin (FOLFOX), 5-FU + leucovorin + irinotecan (FOLFIRI), 5-FU + leucovorin + oxaliplatin + irinotecan (FOLFOXIRI)]. For stage IV, chemotherapy, combinational chemotherapy, and immunotherapy include bevacizumab, ramucirumab, cetuximab, and panitumumab.

Cancers in stage IV have migrated to distant organs and tissues from the colon. The liver is where colon cancer spreads most frequently, although it can also affect the lungs, brain, peritoneum (the lining of the abdominal cavity), or distant lymph nodes. The treatment depends upon the severity of metastases. If the cancer is spread to a few small areas, it can be removed by surgery along with partial colectomy. If the metastases spread to many of the organs, chemotherapy is the primary treatment, and surgery may be an option if the tumor size shrinks [108,109].

**Natural products for CRC treatment:** Recent studies have uncovered the importance of numerous natural products as anticancerous agents because it enhances their quality of life due to their low toxicity and long-lasting nature. Numerous chemotherapeutic agents (almost 50%-60%) are well studied that are obtained from animals, plants, microorganisms, and marine microorganisms to exert anti-cancerous effects [110]. Some of the important natural anticancer agents against CRC are given in Table 1. These natural products are categorized as alkaloids, polysaccharides, polyphenols, terpenoids, and polyunsaturated fatty acids based on their chemical makeup.

Berberine is an isoquinoline natural alkaloid derived from the root, rhizomes, stem, and bark of various plants like *Berberis vulgaris*, European barberry, Oregon grape, and tree turmeric. Berberine inhibits nuclear factor-kappa B and Wnt/ $\beta$ -catenin signaling pathways and exerts an antiproliferative and antiapoptotic effect in cancer. It negatively regulates the action of arylamine N-acetyltransferase in a colon cancer cell line [111] and alters drug resistance by modulating pgp-10 expression in cancer cells [112].

Another alkaloid is irinotecan (CPT-11), a hydrophilic compound derived from camptothecin, which blocks RNA synthesis and prevents DNA synthesis by inhibiting DNA topoisomerase I [113]. CPT-11 effects have been enhanced in CRC when combined with 5-FU/formyltetrahydrofolate [114]. Piperine, an alkaloid of black pepper, enhances the bioavailability, increases absorption, and has favorable effects on the brush border epithelium ultrastructure [115]. Piperine arrests the cell cycle in the G1 phase by inhibiting the expression of cyclin D1 and D3 as well as their dependent kinases 4 and 6 in HT-29 colon carcinoma [116].

Some polysaccharides with anticancer properties, like fucoidan, obtained from seaweed, are a sulfated polysaccharides. Fucoidan plays a major role in suppressing the toxicity of anticancerous drugs

in CRC[117]. Many polyphenols also play a significant role in preventing gastrointestinal malignancies. Curcumin is the most common polyphenol downregulating the gene products implicated in antiapoptosis, cell proliferation, invasion, and angiogenesis[118]. Curcumin shows anticancerous activities in *in vitro* (breast, cervical, colon, gastric, hepatic, leukemia, oral epithelial, ovarian, pancreatic, and prostate cancer cell lines) and preclinical animal models. Five studies of the anticancerous activities of curcumin in CRC were in phase I clinical trials. Each clinical trial found that curcumin is risk-free and has few side effects[119].

Gingerol is another potent polyphenol with various properties like antiemetic, antihyperlipidemic, anti-inflammatory, antioxidant, antiulcer, antihypertensive, immuno-stimulant, and cardiogenic. It regulates multiple cell signaling pathways and their constituents like AP-1, growth factors, p53, COX-2, mitogen-activated protein kinase (MAPK), VEGF, nuclear factor-kappaB, and cyclin D1, which further contribute to cancer initiation and progression[120,121]. In the human colon cancer cell line LoVo, 6-gingerol in a dose-dependent manner reduced the cell viability by arresting the cell cycle in the G2/M phase[122]. A phase II study revealed that andrographolide, a terpenoid, activates various proapoptotic signaling cascades and induces apoptosis (NCT01993472). Polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid have shown the ability to treat malignancies including CRC, breast cancer, and pre-existing adenocarcinoma[123].

**Role of probiotics in CRC:** Probiotics are becoming progressively significant in basic and clinical examination. Studies have established a substantial relationship between probiotics and CRC and how some strains of good bacteria, *i.e.* probiotics, can have a therapeutic and preventive effect against CRC. Studies have shown that probiotics can also lower secondary complications arising from chemotherapies. In a CRC animal model, probiotic supplementation diminished the formation of aberrant crypts, improving the antitumor impact of 5-FU chemotherapy.

Probiotics can influence intestinal physiology either directly or indirectly by modulating endogenous microflora. Previously, researchers cocultured HT29 colorectal carcinoma cells with *Propionibacterium acidipropionici* strain CNRZ80, *Propionibacterium freudenreichii* subsp. *freudenreichii* strain ITG18, and *Propionibacterium freudenreichii* subsp. *Shermanii* strain SI41. These strains were found to have a potent cytotoxic effect[124]. Another great bacterial variety, *Lactobacillus*, is a gut-resident probiotic that benefits host health. A study on *Lactobacillus casei* BL23 significantly protected mice against CRC development; specifically, *Lactobacillus casei* BL23 treatment reduced histological scores and proliferative index values[125]. Another *in vivo* study with *Lactobacillus plantarum* strains YYC-3 and YYCS prevented the occurrence of colon tumors and mucosal damage in APCMin/+ mice fed a high-fat diet. However, YYC-3 had a more robust anticancer effect[126].

Some anaerobic microscopic organisms process prebiotics like oligosaccharides into short-chain fatty acids. Short-chain fatty acids, such as acetate, propionate, and butyrate, have many beneficial effects like improving tight junctions, antiproliferative function[127], and anti-inflammatory function by stimulating immunosuppressive cytokines such as interleukin-10[128]. According to the findings, administering specific fecal bacteria, also known as fecal microbiota transplantation (FMT), into a patient's colon may improve a the response to immunosuppressive medications that increase the capacity of the immune system to identify and eradicate tumor cells. Numerous clinical trials have shown cure rates of about 90% when repeated FMTs are included for the treatment of recurrent *Clostridioides difficile* infection[129,130]. By replacing CRC-associated dysbiosis and restoring eubiosis in chronic illnesses, the adoption of FMT protocols could lessen the activation of inflammatory, proliferative, and procarcinogenic pathways as well as microbiota-induced genotoxicity. Even though fecal transplantation has not been well investigated in CRC, future studies will significantly advance this field of study[131].

**Biomarker-driven therapy:** Conventional drugs are less effective, non-specific, and have more secondary consequences than benefits. Therefore, it is necessary to focus research on a different direction. The following signaling pathways in CRC are dysregulated: APC (Wnt pathway), RAS, RAF, KRAS, RET, MAPK, P53, and SMAD. Figure 2 lists therapeutic agents that target these mutations at particular stages of CRC. CRC shows a sequence of mutations. The very first step in the chromosomal instability pathway begins with the APC mutations. APC is a tumor suppressor protein that leads to familial and sporadic types of CRC. It coordinates with axin,  $\beta$ -catenin, and glycogen synthase kinase-3  $\beta$  and regulates  $\beta$ -catenin in the Wnt signaling pathway. Tasin-1 and statins are drugs that target APC. Tasin-1 induces endoplasmic reticulum stress-dependent jun N-terminal kinase activation and apoptosis in mutated APC human colon cancer cells. It also suppresses AKT activity in a cholesterol-dependent manner[132]. APC mutations are followed by the mutational activation of oncogene KRAS and SMAD-4 and inactivation of tumor suppressor gene p53. About 40%-50% of CRC patients have KRAS mutations.

Mutated RAS enhances the activation of downstream signaling pathway molecules like RAF and MAP kinase. It leads to a malfunctioning GTPase activity and more frequently affects exons 2, 33, and 34. Drugs like tipifarnib, deltarasin, and cismethynil target RAS and KRAS mutations in CRC. Cismethynil inhibits cell growth in the colon cancer cell line in an isoprenylcysteine carboxyl methyltransferase-dependent fashion[133]. SMAD4 is a tumor suppressor protein and an important molecule of transforming growth factor-beta. Truncated and mutated SMAD-4 is involved in tumor progression

and metastasis. It promotes distant metastasis as compared to lymphatic metastasis of CRC[134]. SMAD-4 is targeted *via* the drug vactosertib. In phase I/II (NCT03724851) trials, vactosertib in combination with pembrolizumab significantly decreased the transforming growth factor-beta-related vactosertib responsive gene signature, and the extent of decrease was more significant in responders compared to non-responders[135].

Mutation in *p53* contributes to 35%-40% of sporadic CRC and almost half in all other cancers[136]. *p53* is a tumor suppressor protein involved in various processes like cell cycle, apoptosis, and angiogenesis regulation. A preclinical study on nutlins-3, an Mdm2 inhibitor in the mouse azoxymethane colon cancer model, reduced both cell proliferation and apoptosis[137]. A study on a small molecule PRIMA-1MET, a mutant *p53* reactivator, has shown promising results in restoring the wild-type structure of *p53* and inducing massive *p53*-dependent apoptosis[138].

The CpG island methylator phenotype route of CRC pathogenesis is linked to *BRAF* hypermethylation, which results in truncated *BRAF*. *BRAF* is stimulated by *RAS* and involved in various processes like apoptosis, cell growth, cell proliferation, cell differentiation, cell survival, and cell migration. This mutation is 8%-10% in CRC patients. This is important for the EGFR-mediated cell signaling pathway, MAP kinase pathway, and Bcl-2 expression. The truncated *BRAF* is targeted by vemurafenib and encorafenib drugs, which reduce signaling through the aberrant MAPK pathway. A phase II study (NCT02164916) has shown that addition of vemurafenib improved PFS and the primary endpoint (HR: 0.50,  $P = 0.001$ )[139]. In the phase III CRC study (NCT02928224), encorafenib plus cetuximab improved OS, objective RR, and PFS in previously treated patients in the metastatic setting compared with standard chemotherapy[140].

### **Most promising therapy among new therapeutic approaches**

The aforementioned strategies are innovative methods for creating contemporary cancer treatments, and they are characterized by benefits, including specificity, effectiveness, minimal to no side effects, a higher survival rate, and a lower risk of CRC recurrence. Based on their similarities and differences, these strategies might be grouped. Natural products, probiotics, and oncolytic viral treatments have various anticancer qualities and can be given at any stage of CRC, which is one similarity. However, biomarker-driven therapy and RNA-based therapy target specific molecular entities depending on their expression at a particular stage, making them extremely stage-specific.

The function of therapy, whether diagnostic, prognostic, preventative, or therapeutic, is another commonality in the similarity index. Probiotics and naturopathic remedies serve largely as preventative [141]. However, RNA-based therapy, oncolytic viral therapy, and biomarker-driven therapy have therapeutic potential[93,98-100,142,143]. In addition, RNA-based methods can be used for prognostic and diagnostic purposes[144]. These methods also differ in how they work; for example, certain natural products and RNA-based therapies work at the mRNA level to halt translation by degrading mRNA. Natural products also have several anticancer properties, such as antiproliferative, antiapoptotic, and RNA/DNA synthesis prevention. On the other hand, oncolytic viral medicines cause cellular lysis by inducing systemic antitumor immunity and selective replication within neoplastic cells, which have a lytic effect on the tumor cells. Additionally, probiotics play a preventative role by enhancing tight junction and antiproliferative activity.

Overall, each strategy has its benefits and drawbacks. But RNA-based treatments have drawn more attention, primarily because of their versatility in modifying a wide range of targets and their capacity to target disease genes that were previously inaccessible for manipulation. The inability of the negatively charged RNA to enter negatively charged cellular membranes; poor cellular uptake, non-specificity, and RNA destruction by nucleases in the plasma are a few difficulties that must be addressed[145]. Advances in medicinal chemistry and nanotechnology will help to solve those issues, and RNA-based therapies will become more widely adopted. RNA-based therapies and oncolytic viral therapies hold the most promising potential. One of the main advantages of oncolytic viruses are they can be engineered to target specific tumor cells without damaging healthy cells. The specificity of oncolytic viruses for tumors can be increased by selective genetic modifications such as the expression of viral surface proteins that bind to cellular receptors found only on cancerous cells, deletion of virus fatal genes, and addition of different immunostimulatory genes[97].

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## **CONCLUSION**

### **Conclusion and future directions**

Human genomic, transcriptional, proteomic, and epigenetic information has never been more accessible than now thanks to advances in medical sciences and electronics. Since every patient's TME is unique, a single CRC treatment strategy was never an option. Moreover, individualized therapeutic approaches are required due to tissue heterogeneity. Although conventional cytotoxic drugs are always the first line of treatment for solid tumors, drug resistance causes patients to develop incurable recurring CRC. As a result of these shortcomings, the development of novel approaches with significant benefits and minimal drawbacks as future perspectives is required. Radiotherapy is also a promising option for rectal

cancer patients. But, it has some plausible and long-term toxicity effects on vital organs that must be overcome by modifying radiation intensities[12].

Chemotherapy has become the mainstay of CRC treatment due to studies conducted in recent decades showing that utilizing it has increased the OS time of CRC patients, particularly those with metastases, to approximately 20 mo[20]. Chemotherapy has several drawbacks, including systemic toxicity, an unsatisfactory RR, fever, mucositis, stomatitis, leukopenia and thrombocytopenia[21], and a lack of tumor-specific selectivity. Recently, this has led to the idea of molecular targeted therapy for CRC.

Various monoclonal antibodies against angiogenic factors/receptors have been developed, including anti-EGFR/EGF, anti-VEGFR/VEGF, and ICIs (CTLA-4, PD-1/PD-L1, TIM-3, anti-LAG-3), which act directly on cancer cells by boosting immune cells such as T cells and NK cells. The impediment of antiangiogenic treatments is that they make cancers impervious to them. Tumor-associated fibroblasts continuously secrete proangiogenic growth factors like EGF, insulin-like growth factor, and particularly platelets-derived growth factor-C, which is a key factor for maintaining angiogenesis even in the presence of antiangiogenic therapies[146]. To address these issues and treat this fatal cancer, more target-specific treatment methods, such as immunotherapeutic and target-specific approaches, including ACT therapies[60], vaccines, and cytokine-based therapies[66], are required. ACT, vaccines, and cytokine-based therapies can defeat the inadequacies raised because of chemotherapeutic drugs as they have the ability to increase and reactivate effector NK and T lymphocytes, promote lymphocyte infiltration of tumors, and persist in the TME. Therapeutic cancer vaccines have the potential to be as effective as monotherapies when used in premalignant cancer and carcinoma in situ. Many cytokine therapy trials are currently ongoing for various cancers[82]. But for CRC, there is a need for preclinical studies to assess their unexpected toxicity.

Along with the aforementioned approaches, new emerging approaches for treating CRC, including RNA interference, oncolytic viral therapy, use of natural products, probiotics, and biomarker-driven therapy, have shown promising results. RNA interference has been proposed as another remedial approach that offers significant benefits over customary medicines, with high explicitness and intensity and low toxicity[147]. Utilizing genetically altered oncolytic viruses is another remedial strategy. Currently, only one oncolytic virus therapy has been FDA approved to treat cancer. T-VEC (Imlygic®) is a modified herpes simplex virus that targets tumor cells and aids in their demise. It has been approved for specific melanoma patient subsets. Numerous studies have shown that many natural products have potent anti-CRC effects and could replace chemotherapy agents in treating CRC.

Furthermore, the composition of microbiota appears to influence the development of CRC. As per scientific literature, utilizing probiotics can assist with forestalling CRC growth[148] by utilizing anticarcinogenic activity *via* potential biologically host-dependent and strain-specific physiological mechanisms[141]. The time has come to modify the available therapeutic approaches and foster novel methodologies with negligible drawbacks and higher advantages for CRC treatments.

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## FOOTNOTES

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**Author contributions:** Kumar A performed the conceptualization, wrote the original draft preparation, and contributed to software, visualization and investigation, and reviewing and editing; Gautam V, Sandhu A, and Sharma A wrote the original draft preparation; Rawat K wrote the original draft preparation and performed the proofreading; Saha L contributed to the conceptualization, supervision, and reviewing and editing.

**Conflict-of-interest statement:** All the authors report no relevant conflicts of interest for this article.

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**S-Editor:** Fan JR

**L-Editor:** Filipodia

**P-Editor:** Fan JR

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