Emerging molecular targets in Metastatic Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of the most frequent cancers in both men and women with an increasing incidence in young adults. Despite the expansion of our understanding regarding the biology and pathogenesis, the advances in the treatment of metastatic CRC disease remain limited. Over the past years, a growing number of molecular targets have attracted the interest of the scientific community. Until now, clinical utility has been confirmed for a numerous of these actionable targets. So, new treatment approaches have focused on angiogenesis and immunotherapy as well as novel inhibitors have been developed against EGFR (Epidermal Growth Factor Receptor), KRAS (KRAS Proto-Oncogene, GTPase), BRAF (B-Raf Proto-Oncogene, Serine/Threonine Kinase), HER2 (Erb-B2 Receptor Tyrosine Kinase 2), NTRK (Neurotrophic Receptor Tyrosine Kinase) and others. In this review, we summarize current knowledge on the validated as well as emerging molecular targets in the treatment of metastatic CRC.

Key words: *Colorectal cancer; targets; treatment*

INTRODUCTION

Colorectal cancer (CRC) appears to be the third most commonly diagnosed cancer and one of the predominant causes of cancer-related mortality worldwide, ranking second following lung malignancies. According to the 2020 GLOBOCAN statistics, there have been recorded approximately more than 1.9 million new cases of colorectal cancer (9.8 % of all cancer cases) along with 935,000 deaths (9,2% of total cancer related deaths.), affecting both men and women [1]. Similarly, the American Cancer Society expects 104,270 new cases of colon cancer and 45,230 new cases of rectal cancer, by the end of 2021 in the United States [2].

The incidence of this cancer type has been increasing accordingly to the human development index with the European countries, Australia and Northern America ranking first in the list. This phenomenon has been related to western-type dietary patterns and lifestyle factors, such as smoking and excess meat consumption [3]. But despite the fact that the survival rate has been improving through the years, especially due to the widely used screening methods, changes in certain daily habits but also the evolution of therapeutic strategies, the overall 5-year survival rate, which mainly depends on the stage of the disease, remains at 65-70% in localized and regional stage cancers but drops significantly below 20% for those whose cancer has spread to distant parts of the body (14% for colon cancer and 16% for rectal cancer according to the American Cancer Society's (ACS) data from 2010-2016.

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Until now, the cornerstone in the treatment of early-stage CRC is the surgical resection of the tumor. However, surgery is rarely a curative option for almost 20-25% of patients that present initially with metastatic cancer or develop later metastatic disease. In these cases, radiotherapy, chemotherapy and most recently immunotherapy are implemented as treatment options.

The heterogeneity of CRC, regarding especially the distinct molecular profile that each tumor has, and therefore its unique clinical features, has generated the necessity to seek alternative treatments. Focusing on molecular targets, these therapeutic strategies are mainly associated with predictive biomarkers, such as microsatellite instability (MSI), mutations in *RAS* (RAS Proto-Oncogene, GTPase) and *BRAF* (B-Raf Proto-Oncogene, Serine/Threonine Kinase) genes, amplification of *HER2* (Erb-B2 Receptor Tyrosine Kinase 2) as well as *NTRK* (Neurotrophic Receptor Tyrosine Kinase) gene fusions and aim to achieve a prolonged survival rate for patients with CRC with significantly less side effects than chemotherapy [4]. An alternative modern treatment option of metastatic CRC is the administration of immunotherapy and especially the immune checkpoint inhibitors which aim to reactivate the immune system response against cancer [5]. In this review, we present current knowledge regarding the molecular targets in the treatment of CRC.

Significant signaling pathways in colorectal cancer

The signaling pathway of the EGFR (epidermal growth factor receptor) has a central role in CRC. The activation of EGFR triggers the activation of PI3K (Phosphoinositide-3-kinase) and MAPK (Mitogen-Activated Protein Kinase) signaling pathways, which constitute significant pathways for cell proliferation, growth and apoptosis inhibition [6,7]. EGFR is present on the cell membranes, while elevated expression levels can be found in neoplastic cells and moderate adenomas [8]. Studies have shown that 60-80% of colorectal tumors have overexpressed EGFR [9].

More specifically, EGFR, a member of the ErbB family of receptor tyrosine kinase, is a transmembrane glycoprotein with an intracellular domain functioning as a tyrosine kinase and an extracellular ligand-binding domain [10]. The ErbB family consists of four ErbB members: ErbB1 (EGFR/HER1), ErbB2 (Neu/HER2), ErbB3 (HER3), and ErbB4 (HER4) [11]. After the ligand binding on the receptor, homo- or hetero-dimerization occurs, phosphorylation of the tyrosine kinase domains is triggered, and the MAPK cascade is activated [10]. Then, the next step of the signaling cascade is the activation of RAS protein. There are three isoforms of Ras GTPases (Guanosine triphosphate) including H-Ras, N-Ras, and K-Ras [12,13].

The RAS protein has two forms, the active GTP bound state, and the GDP (Guanosine diphosphate) bound state [14]. RAF activation by phosphorylation is mediated by active RAS leading to MEK/MAPK (Mitogen-activated protein kinase) activation as well as to phosphorylation and activation of ERK (extracellular signal-related kinase) [15–17]. Phosphorylated ERK translocates from the cytoplasm to the nucleus, as a transcription factor, phosphorylates and regulates various other transcription factors, including carbamoyl phosphate synthetase II (CPS-II) and p90RSK. The final result is the expression of target genes (e.g. *c-FOS, c-JUN and myc*) by the transcription factors, leading to cell survival and growth [18,19].

Role of angiogenesis in colorectal cancer

Angiogenesis is the process through which new vascular networks originate and branch from pre-existing vessels. It takes place mainly during early embryogenesis, while in adults blood vessels rarely form new brunches, except in tissue repair or disease conditions, including cancer progression. It involves the migration of endothelial cells at the lead of growing vessels, lumen formation and the maturation of newly formed blood vessels through the recruitment of mural cells and the consolidation of cell to cell adhesion [20]. The rapid development of new vascular networks is necessary to support the progression of cancer and therefore sustain neoplastic growth, while these events also facilitate the dissemination of metastases [21]. The most important angiogenic regulators are the vascular endothelial growth factor (VEGF) and its receptors, which are overexpressed in metastatic CRC [22]. Other mediators of angiogenesis include platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [23].

The VEGF family consists of five secreted glycoproteins (termed VEGF-A to -E) and the placenta growth factor (PlGF)-1 and -2, which bind, with different affinity and specificity, to three receptor tyrosine kinases (RTKs) on endothelial cells (termed VEGFR-1 to -3) [24,25]. VEGF promotes angiogenesis in several ways, which are mediated by intracellular signaling events initiated by the binding and dimerization of cognate receptors on endothelial cells. It has been shown that VEGF can induce vascular permeability through ERK1/2 (Extracel-

lular Signal-Regulated Kinase 1/2) and AKT signaling pathways, thus creating a pro-angiogenic environment [26]. Moreover, VEGF leads to upregulation of the anti-apoptotic BCL2 protein through the PI3K/AKT pathway and confers a survival signal to endothelial cells, while induces the secretion of key enzymes, such as metalloproteinases and other proteases, necessary for the migration and invasion of endothelial cells [27–29].

Upregulation and secretion of VEGF in the tumor microenvironment are mostly driven by hypoxia, which is the result of insufficient vascular supply inside the growing tumor. The hypoxia-inducible factors (HIFs) are upregulated during hypoxic conditions leading to the transactivation of angiogenesis-related genes (VEGF, PDGF-B) and cell proliferation (TGF-β) [30]. Besides hypoxia, HIF proteins are also upregulated through specific oncogenic signaling effectors, including ERK and PKA [31,32]. The production of VEGF can also be directly promoted by the activation of oncogenes, including *KRAS*, *HER2*, *EGFR* or members of the MAPK cascade, all of which can be found mutated in CRC [33–36]. Finally, VEGF expression can be mediated by several growth factors and cytokines, such as PDGF, IGF and prostaglandins [37–39].

Tumor vascular networks demonstrate high degrees of heterogeneity and atypical morphological features compared to normal vasculature. They are characterized by excessive permeability, poor perfusion and disorganized vascular pressure due to vascular immaturity and mechanical forces applied on the vessels by the growing tumor [40,41]. These events result in hypoxic areas that drive cancer cells to acquire a more aggressive phenotype [42]. In addition, numerous studies have documented the role of angiogenesis in colon cancer progression and metastasis, since it provides a conduit for cancer cell dissemination [43]. In this vein, not only the VEGFR-VEGF axis, but also other mechanisms, such as Notch signaling activation and E-selectin expression, are recruited [44,45]. Moreover, this heterogeneity has a direct impact on the efficacy of the available treatment options. Cells under hypoxic conditions are less sensitive to radiation, while insufficient blood supply of specific areas inside the tumor limits the delivery of chemotherapeutic agents and host immune cells triggered by immunotherapies to target cancer cells [46–48].

Targeting angiogenesis in colorectal cancer

Targeting angiogenesis is a major approach in cancer treatment. Although in metastatic CRC and most other cancer types, angiogenesis is not a determinant of cancer progression, anti-angiogenic treatment options show significant clinical activity [49]. Besides the reduction of tumor growth and inhibition of metastasis, they can also normalize vascular permeability and facilitate the delivery of chemotherapeutic agents, resulting in more effective cancer treatment [50].

The overall clinical benefit has been well established, even if it is slight. The two categories of medicines that target angiogenesis include monoclonal antibodies (mAbs) and tiny chemicals, such as tyrosine kinase inhibitors (TKIs) [51]. The mAbs act by either directly binding to VEGF-A or blocking the appropriate receptor's extracellular binding domain. Three mAbs are used in clinical practice: bevacizumab, aflibercept, and ramucirumab. Bevacizumab is a humanized IgG monoclonal antibody that binds to all isoforms of VEGF-A. Aflibercept is a soluble decoy receptor that binds to VEGF and stops it from activating its native receptors. Ramucirumab binds with a high affinity to the VEGFR-2 extracellular domain, preventing VEGF ligands from binding and thereby blocking receptor activation. TKIs work by binding to and inhibiting the kinase domains of a variety of receptors involved in the angiogenesis process [51,52].

Angiogenesis can be targeted for the management of mCRC in any line of treatment. Bevacizumab has been combined with chemotherapy in both first and second line of treatment, while aflibercept and ramucirumab have been approved for second-line treatment. In addition, regorafenib (TKI) is used as monotherapy in patients with chemo-resistant illness [53,54].

Bevacizumab is the most widely used anti-angiogenic inhibitor. Since monotherapy has a minor effect, it is frequently used with chemotherapy to improve efficacy as evaluated by the response rate (RR), progression-free survival (PFS), and overall survival (OS). It has been shown that when paired with chemotherapy, it outperforms the chemotherapy plus placebo [55–60]. Bevacizumab is used in conjunction with modern combination therapies. It appears to be more effective with the triplet FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) than with FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) alone [61]. After first-line treatment, bevacizumab is also effective when paired with chemotherapy [62–64]. Unfortunately, there are no clinically validated biomarkers for predicting bevacizumab benefit. In addition, bevacizumab can cause vascular adverse effects, the most dangerous of which

are gastrointestinal perforation, bleeding, and arterial thrombosis (<1% of patients). Furthermore, proteinuria, hypertension, and leukopenia are also common side effects [65,66].

In addition, ramucirumab is an approved medication for the second-line treatment of mCRC. In comparison to FOLFIRI alone, the combination of ramucirumab with FOLFIRI increased PFS and OS but not response rate [67]. On the other hand, in a phase II trial, it was discovered that adding ramucirumab to the FOLFOX (fluorouracil-leucovorin-oxaliplatin) regimen did not improve PFS [68]. Furthermore, aflibercept binds to VEGF-A more effectively than bevacizumab [69]. When combined with FOLFIRI, aflibercept improves survival in patients who had previously progressed on an oxaliplatin-containing therapy [70]. In the first-line scenario, however, the combination of aflibercept and FOLFOX did not produce any apparent improvement. As a result, aflibercept is used as a second-line CRC treatment [71].

Anti-angiogenic TKIs have also been evaluated in people with mCRC. Regorafenib is the only TKI that is used in the clinical practice of mCRC. CORRECT trial confirmed a survival benefit for regorafenib as monotherapy (median OS 6.4 months) compared to placebo group (5 months) [72]. A similar benefit over regorafenib (median OS 8.8 vs 6.3 months) was also confirmed in the CONCUR phase III clinical trial, in which only Asian patients were recruited [73]. Rash, fatigue, hand-foot skin response, anorexia and diarrhea are the most common adverse reactions in patients treated with regorafenib, and dose reductions are frequently required to manage regorafenib-related adverse events. A lower initial dose with a gradual dose increase has been proven in several trials to be an alternate, safe, and well-tolerated route to regorafenib administration, and this approach should be favored in daily practice [74,75].

RAS/RAF wild type and anti-EGFR therapies

The increased presence of the EGFR on cancerous tissue of the colon and rectum was detected about 35 years ago [76]. Since then, great progress has been made in the understanding of its involvement in disease pathogenesis, while two targeted biological agents have been approved and are widely employed in clinical practice.

Cetuximab and panitumumab represent the only approved anti-EGFR targeted therapies for metastatic colorectal cancer, with equivalent efficacy [77]. They are monoclonal antibodies that either bind extracellularly and downregulate EGFR and, subsequently, its tumor-promoting signaling or induce cancer cell death by mediating antibody-dependant cytotoxicity (ADCC) [78]. They also display a synergistic effect in combination with chemotherapy. In randomized controlled trials on the metastatic setting of colorectal cancer, cetuximab monotherapy increases overall and progression-free survival in chemotherapy pre-treated patients [79], while its addition to the pre-existing fluorouracil plus irinotecan combination can be used as first-line to reduce progression risk [80]. Similarly, adding panitumumab to fluorouracil/leucovorin plus oxaliplatin results in longer progression-free survival [81].

Previous and ongoing research on other anti-EGFR strategies has yielded mixed results. EGFR tyrosine kinase inhibitors have been hypothesized to inhibit EGFR-regulated pathways, as in the case of KRAS-wt Non-Small Cell Lung Cancer (NSCLC) [82]. Their success however was not repeated in early trials of gefitinib plus chemotherapy [83,84], while erlotinib has been proven more promising in increasing survival in KRAS-wt metastatic colorectal cancer [85], but these results were not consistent with those of other studies [86]. High toxicity was the common denominator among all studies [83–87].

EGFR itself is less important as a predictive marker of response and anti-EGFR therapies are indicated regardless of its degree of expression [88,89]. On the other hand, the absence of KRAS mutations, especially in exon 2, is a prerequisite for the administration of anti-EGFR targeted therapy, which is otherwise not only ineffective [90,91] but has been shown to expedite terminal outcomes [81]. This is attributed to bypassing EGFR signaling and activating the RAS/RAF/MAPK signaling pathway, enabled by the mutant variants [78]. Similarly, human epidermal growth factor receptor 2 (HER2) amplification is associated with shorter progression-free survival [92], possibly due to the EGFR-independent downstream activation of PI3K/AKT/mTOR and RAS/RAF/MAPK cascade or by heterodimerization with EGFR [93].

Interestingly, the location of colon cancer is of prognostic and predictive value. Based on data from the CRYSTAL [80] and FIRE-3 [94] randomized controlled trials, patients with left-sided RAS-wt metastatic colon cancer clearly benefit more from cetuximab plus chemotherapy in terms of response rates and survival than patients with right-sided tumors [95]. Right-sided tumors generally have a worse prognosis regardless of the interventions used [96] and display different histopathological and molecular characteristics compared to left-sided tumors [97],

including less robust EGFR signaling, that could explain the inefficiency of anti-EGFR strategies.

KRAS as a target

As we mentioned above, RAS is a protein family of 3 members KRAS (Kirsten rat sarcoma virus), NRAS (neuroblastoma RAS), and HRAS (HRas Proto-Oncogene, GTPase) that have GTPase function at the signal transduction of most growth factor receptors such as the EGFR [98]. *RAS* activating mutations and especially *KRAS* mutations are the most common genetic alterations in human carcinomas, accounting for almost one million deaths every year worldwide. It is found in about 40% of CRCs and has an anatomic specificity to the more aggressive right-sided tumors as compared to left-sided ones that are more likely to have *EFGR* mutations [99]. Mutations mostly in codons 12, 13, 61 in the *RAS* gene result in different *KRAS* mutant alleles with the most common ones for CRC being *G12D*, *G12V*, *G13D*, *G12A*, *G12S*, and *G12C*. The majority of the above mutations are caused by a single amino acid substitution.

Therapies targeting KRAS would be very effective for colorectal malignancies but unfortunately creating such an inhibitor is rather difficult and none has yet been approved. For now, the only drugs inhibiting KRAS directly are sotorasib (AMG510) and adagrasib (MRTX849). They bind to the P2 pocket of the switch I/II region of KRAS and lock it in its inactive form. Sotorasib is only approved for patients with advanced or metastatic NSCLC positive to the KRAS G12C mutation that have been previously treated with at least one other therapy [100]. Currently, multiple clinical trials in phases I and II examine the use of sotorasib in CRC as well. Specifically, the CodeBreak100 trial [101] ended up to the conclusion that by using sotorasib, malignancies can be controlled, and patients may benefit with up to 5.4 months of stable disease duration. The Krystal-1 trial [102] concluded that Adagrasib can also have therapeutic effects on patients with CRC, especially when combined with anti-EGFR treatments. Another compound that is being studied is BI-2852 that also binds at the same region of KRAS G12C mutation making it unable to bind with SOS1 and its effectors PI3K and RAF at different doses. Both MAPK and PI3K/AKT pathways were blocked producing antiproliferative effects in mutant cells. These studies confirmed that KRAS can be targeted directly and research in that direction should continue.

A recent study came up with a SOS1 (Son of Sevenless) inhibitor (BI-3406) [103] that blocks the binding of SOS1 to RAS when *KRAS* alleles *G12* (especially *G12D, G12V, and G12C*) and *G13D* as well are present. As a result, RAS cannot be activated leading to the blockage of cell proliferation. The combination of BI-3406 with a MEKI also known as MAPKI (Mitogen-activated protein kinase inhibitor) I- trametinib was also tested. The discovery of this compound and other analogs like BI 1701963 (phase I clinical trial NCT04111458) show a very promising therapeutic potential.

B Raf Kinase (BRAF) as a target

BRAF protein is a member of a serine-threonine kinase family. RAF kinases act mainly through phosphorylation and play an important role in many cellular processes, such as cell proliferation, differentiation and regulation of transcription. The proto-oncogene *BRAF*, which encodes the corresponding serine-threonine protein kinase, plays a critical role in the carcinogenesis not only of colorectal cancer, where it is mutated in 5% -8% of cases, but also in many other forms of the disease [104]. In particular, mutations in the gene can either be inherited or appear later in life and cause cancer [105]. Gene mutations show great diversity and many have been detected in large numbers (over 30) [106]. These can explain the development and progression of many malignancies. Typical examples are melanoma, NSCLC, colon cancer, papillary thyroid carcinoma, glioblastoma and astrocytoma [104,107,108].

Mutations may occur in different regions of the gene sequence. However, the most commonly identified mutations in colorectal cancer (and other malignancies) involve the replacement of thymine by adenine at nucleotide 1799, leading to the replacement of valine (V) by glutamate (E) at code 600 [108]. This mutation typically affects women, older people, smokers and more often right colon cancers [106,109]. Obviously, this mutation can play a crucial role in patients' prognosis and response to treatment. This is because it usually involves low-grade, advanced cancers that have already had lymph node metastases and perineural infiltration [109]. In addition, this mutation is associated in some studies with poor prognosis and may adversely affect patients with hepatic and pulmonary metastasectomy [107,110,111]. It is also negatively associated with the response to anti-EGFR agents [109]. Other mutations which have been found are G463E, G463V, G465A, G465E, G465V, G468A, D593V, F594L, etc. [112].

Before the era of BRAF targeting, intensive chemotherapy combined with anti-VEGF therapies was the

most appropriate approach for patients with BRAF-V600E [113]. However, during recent years, numerous of studies, which have evaluated different BRAF inhibitors as well as different combinations, have provided adequate evidence regarding the clinical significance of BRAF blockade in colorectal cancer. It is well documented that monotherapy with a BRAF-V600E inhibitor doesn't improve significantly response rates as well as that only simultaneously targeting at multiple steps provides clinically significant results. So, different combinations of BRAF inhibitors with anti-EGFR monoclonal antibodies and/or MEK inhibitors seem to have achieved the most promising results. Briefly, according to the BEACON trial, the combination of encorafenib (BRAF inhibitor) and cetuximab with or without binimetinib (a MEK inhibitor) in pre-treated patients with metastatic CRC improved clinical outcomes with a tolerable toxicity [114]. In addition, the use of vemurafenib (another BRAF inhibitor) together with cetuximab and irinotecan in patients with metastatic CRC has also showed positive results in terms of response to treatment and progression-free survival [115]. Furthermore, G. Middleton et al. have reported that the dabrafenib-trametinib-panitumumab combination produces a relatively satisfactory response in patients with BM1 gene expression profile, which represents 30% of all BRAF-V600E mutant CRC. These patients are characterized by increased potential for metastasis, activation of KRAS/AKT (AKT Serine/Threonine Kinase) signaling, stronger immune response and resistance to chemotherapy. On the other hand, in patients with BM2 disease (V600E mutation, deregulation of cell cycle control points, enrichment in metabolic processes), it was less beneficial [116]. Many other combinations have been studied or are under investigation, while other open questions are the best sequence strategy as well as the significance of the combination of target therapy with immunotherapy [113].

Microsatellite instability-high (MSI-H) tumors

The DNA replication mechanism is well conserved and maintained mainly due to the DNA polymerase's activity. However, the enzyme cannot always detect and repair its errors. In such a case, the MMR (mismatch repair) mechanism, among others, plays a crucial role [117]. Throughout the human genome, there are many short repetitive loci of DNA, called STRs (short tandem repeats) or microsatellites, which consist of one to six nucleotide repeats [118]. The term MSI refers to the presence of altered microsatellites' length (either longer or shorter) due to a defective MMR mechanism caused by mutations or epigenetic changes in one of its fundamental genes such as MSH2 (MutS Homolog 2), MSH6 (MutS Homolog 6), MLH1 (MutL Homolog 1), PMS1 (PMS1 protein homolog 1) and PMS2 (PMS2 protein homolog 2) [119]. In 1997, the American National Cancer Institute (NCI) suggested the classification of MSI status based on the number of detected mutated loci. More specifically, five significant loci used as biomarkers (BAT 25, BAT 26, D2S123, D5S346, and D17S250) are being examined and tumor status is classified as follows: MSI-H (Microsatellite Instability High) in the presence of two or more mutations detected, MSI-L (MSI low) when there is one and MSS (Microsatellite stable) when there is none [120].

MSI is considered as a hallmark of Lynch Syndrome (LS). Approximately 90% of LS cases are characterized by germline mutations of MMR genes, inherited in an autosomal dominant manner. LS predisposes to carcinogenesis at a younger age (before 50 years old) mainly of the colon, stomach and endometrium [121]. Screening of LS includes testing for MSI based on the Amsterdam Criteria [122] or the Bethesda Guidelines [123]. However, it is known that MSI does not take place early during tumorigenesis and only half of the colon adenomas examined will test positive [124], being indicative for LS since it is a rare finding in sporadic CRC. MSI is relatively frequent in sporadic CRC since 15% of the cases show deficient MMR (dMMR) mechanism. Hypermethylation of the MLH1 promoter constitutes the most common cause of dMMR sporadic CRC. This epigenetic alteration is often combined with BRAF V600E mutation but only in MSI-H sporadic CRC. Hence, when such a situation is identified, it contributes to the differential diagnosis between sporadic CRC and LS [125].

The MSI-H phenotype appears to have distinctive clinicopathological and histological features compared to MSI-L and MSS. These tumors mostly arise in the proximal colon (right-sided location) and are characterized by poor differentiation, high tumor infiltrating lymphocytes (TILs) counts and mucinous cell type [126]. An early-stage diagnosed MSI-H CRC has a favorable prognosis in comparison with MSS or MSI-L. Prominent lymphocytic infiltration of an MSI-H tumor suggests a high antitumor immune response leading to apoptosis, a result that probably explains the improved prognosis [127]. Nowadays, the MSI status of a CRC contributes to a more individualized selection of therapy. Ribic et al. concluded that MSI-H CRC do not benefit from fluorouracil-based adjuvant chemotherapy [128].

Immunotherapy in microsatellite instability-high (MSI-H) patients

The three primary treatment methods of metastatic colorectal cancer (mCRC), surgery, chemotherapy and radiotherapy remain the standard of care but their benefits have already reached a plateau. Therefore, it is urgent to develop a new effective therapeutic strategy to improve the survival outcome of cancer patients. At present, immunotherapy and targeted therapy are promising treatment strategies for CRC, since they have the potential to provide improved therapeutic efficacy with limited toxicity. Following the successful results of immunotherapy in other types of cancer, the interest in its use in CRC is highly increased and continuously growing [129].The way immunotherapy fights cancer is by stimulating the immune system against tumors. There are several categories of immunotherapy for many cancer types: adoptive cell therapy, cancer vaccines, oncolytic virus therapy, targeted antibodies, immunomodulators, etc. To date, immunomodulators have already been approved by the Food and Drug Administration (FDA) for the treatment of patients with dMMR/MSI-H mCRC and seem to be the most promising solution. One of the main representatives of immunomodulators are immune checkpoint inhibitors (ICIs) which regulate the interaction between T cells, antigen-presenting cells (APCs), and tumor cells to boost the release of suppressed immune responses. ICIs target co-inhibitory receptors, such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) expressed on T-cells and other immune-cell subpopulations, or their ligands, such as programmed cell death protein 1 ligand 1 (PD-L1) expressed on tumor cells and various immune cells [130]. The very high mutation rate in dMMR/MSI-H mCRC leads to the production and accumulation of hundreds of somatic mutations which results in a highly effective neoantigen presentation that attracts T-effector cells, such as CD8+ TILs, T helper 1 (Th1) CD4+ TILs and macrophages, as well as immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) and T-regulatory (Tregs) cells. In addition, these tumor cells exhibit upregulation of several immune checkpoint regulators such as PD-1, PD-L1, CTLA-4, Lymphocyte activation gene 3 (LAG3). That explains the high response rates and high sensitivity observed in dMMR/MSI-H mCRC patients treated with ICIs [131].

Two early phase II studies, KEYNOTE-016 and 164 evaluated single-agent pembrolizumab (anti-PD1) in previously treated dMMR/MSI-H mCRC. Patients in KEYNOTE-164 were divided into two cohorts, ≥2 (cohort A) or ≥ 1 (cohort B) prior lines of therapy (fluoropyrimidine, oxaliplatin, irinotecan, or anti-VEGF/EGFR). Pembrolizumab, at a flat dose of 200 mg every 3 weeks, was administered with a disease control rate (DCR) of 51% and 57% for cohorts A and B, respectively. The immune-related objective response rate (ORR) was 33% (N=124) [132]. On the other hand, in KEYNOTE-016 patients were divided into three separate cohorts: dMMR/MSI-H CRCs, pMMR/MSI-L CRCs, and dMMR/MSI-H non-CRCs, they were administered with a 10 mg/kg dose of pembrolizumab every 14 days. This study highlighted the different activity of pembrolizumab in CRC based on MMR status; the PFS at 20 weeks was 78% in dMMR/MSI-H CRC vs 11% in pMMR/MSI-H CRC and the ORR was 40% and 0%, respectively. An interesting point of this research was that the number of somatic mutations was significantly correlated with the chance of achieving response to therapy [133,134]. Pembrolizumab showed significant efficacy in the refractory setting following chemotherapy in patients with dMMR/MSI-H mCRC and was approved by the U.S. FDA for this indication, in 2017.

KEYNOTE-177 is a phase III, open-label trial, whose results led to the approval of pembrolizumab as monotherapy for the frontline treatment of patients with unresectable or metastatic, dMMR or MSI-H CRC. In this study, investigators compared the efficacy of first-line pembrolizumab monotherapy (N=153) vs standard of care chemotherapy ± bevacizumab or cetuximab (N=154) in 307 patients affected by dMMR/MSI-H mCRC. Pembrolizumab was superior to standard chemotherapy in terms of PFS (median 16.5 months vs 8.2 months, hazard ratio 0.60, 95% CI, 0.45-0.80, p = 0.0002) with a lower rate of treatment-related adverse events (AEs) (G3-5 AEs 22% versus 66%). Also, the ORR was 43,8% vs 33,1% for pembrolizumab and chemotherapy, respectively [135,136].

Immune checkpoints have been found to be overexpressed in dMMR/MSI CRCs compared to pMMR/MSS CRCs [137]. Then, combinations of monoclonal antibodies should be a solution to avoid primary resistance of dMMR/MSI-H mCRC to pembrolizumab. The FDA also approved nivolumab (anti-PD1) either alone or in combination with low dose ipilimumab (anti-CTLA4), for patients with dMMR/MSI-H mCRC, based on the results of the CheckMate 142 study. In this study, the researchers assessed the efficacy of nivolumab as first-line monotherapy (N=74) in comparison with the

combination of nivolumab with ipilimumab (N=119) and demonstrated very promising results, ORR 23% vs 55%, DCR 69% vs 80% and G3-4 AE rates 21% vs 32%, respectively [138,139]. Several ongoing clinical trials investigate the impact of the combination of immunotherapy and chemotherapy in dMMR/MSI-H mCRC patients, such as the COMMIT study which has 3 treatment arms, atezolizumab (anti-PD-L1) monotherapy vs FOLFOX + bevacizumab (anti-VEGF) vs atezolizumab + FOLFOX + bevacizumab [140]. Similarly, CheckMate 8HW is an ongoing study of nivolumab with or without ipilimumab or investigator's choice chemotherapy in dMMR/MSI-H mCRC patients [141]. Moreover, avelumab (anti-PD-L1) is investigated as an option in the second-line setting. In the SAMCO study avelumab is compared with standard of care in dMMR/MSI-H mCRC patients [142].

At present, many clinical trials investigate the efficacy of ICIs in combination with targeted therapies such as anti-VEGF drugs, anti-EGFR drugs, MAPK pathway inhibitors and multitarget kinase inhibitors. Moreover, the modulation of gut microbiota or fecal microbiota transplant seem to be promising options for boosting immunotherapy, in patients with dMMR/MSI-H mCRC with secondary resistance to ICIs (NCT03775850) [143]. Moreover, vaccination with frameshift peptides is one more option as a strategy to exaggerate primary or secondary resistance to ICIs in these patients (NCT04041310) [144–146].

Human epidermal growth factor receptor 2 (HER2) as a target

HER2 amplification occurs in 5% of mCRC patients [147]. The clinical significance of HER2 regarding its prognostic value in CRC needs further clarification. Early studies proposed a negative prognostic impact of HER2 overexpression, but more recent trials didn't confirmed the association between HER2 amplification and outcome [148,149].

According to the PETACC3 adjuvant chemotherapy trial and the subsequent DNA copy number & gene expression analysis, proximal carcinomas (ascending, hepatic flexure, transverse colon) were less likely to be HER2 or EGFR amplified compared to distal carcinomas (splenic flexure, descending colon, rectum) [97]. HER2 amplification in mCRC is enriched in KRAS, NRAS, BRAF and PIK3CA WT tumors and is a resistance marker for EGFR antibody therapy [150]. HER2 positive patients show more frequently lung metastases and higher tumor burden as well as HER2 positive tumors were more likely to be left sided [151]. Furthermore, HER2 status is also a molecular predictive biomarker for anti-HER2 targeted therapies (Trastuzumab/pertuzumab or Trastuzumab/lapatinib) [152].

The clinical significance of HER2 amplification regarding HER2-targeted therapies in patients with mCRC has been confirmed in many clinical trials (Figure 1). TRIUMPH was a phase II trial, in which circulating tumor

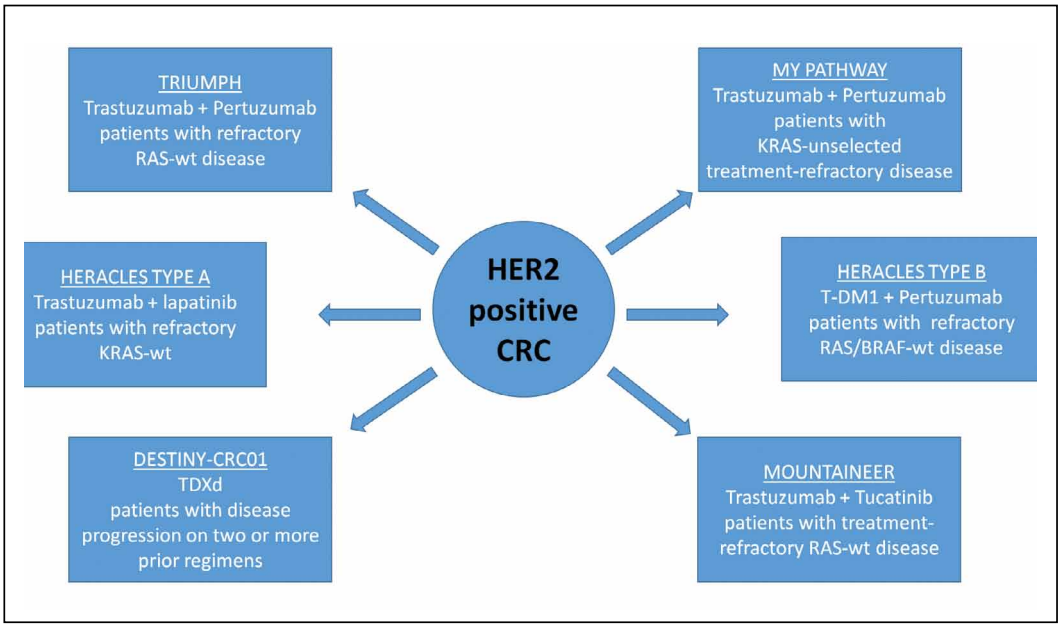

Figure 1. Clinical trials in which anti-HER2 treatments have been evaluated.

DNA (ctDNA) and simultaneously tissue HER2 testing were used [153]. The aim was to recognize patients for dual-HER2 blockade treatment with pertuzumab plus trastuzumab in patients with HER2 amplification and RAS wild-type. The outcomes of this investigation with pertuzumab / trastuzumab as a treatment in patients with chemorefractory RAS-WT disease (n=18) are ORR 35% (tissue positive), 33% (ctDNA-positive) and median progression free survival (mPFS) at 4 months. Findings from the TRIUMPH study confirmed the usefulness of ctDNA as a screening platform to select patients with HER2-amplified mCRC who will benefit from dual-HER2 blockade with trastuzumab and pertuzumab. The drawbacks of TRIUMPH are the small sample size and the use of a registry control arm [153].

In HERACLES-A trial, fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) were used. In this study, the above diagnostic algorithms were utilized to screen HER2-positive tumors for therapeutic targeting. More specifically, few patients had histologically confirmed KRAS-WT exon 2 (codons 12 and 13) and HER2-positive mCRC. Dual HER2 blockade with the combination of trastuzumab and oral lapatinib until disease progression or toxicity were investigated. The outcomes were ORR 28%, mPFS at 4.7 months in patients with increased gene copy number (GCN) > 9.5 and 3.7 months in patients with HER2 GCN <9.5 with median OS 10.0 months. The long-term (6.7 years) follow-up analysis of HERACLES-A provides strong evidence that the administration of trastuzumab and lapatinib combination in KRAS wild-type, chemorefractory HER2-positive mCRC patients provide survival as well as clinical benefits [154].

Another significant study was HERACLES-B study, which evaluated a targeted approach with a combination of pertuzumab and trastuzumab-emstasine (T-DM1) [155]. It was a single-arm, phase II trial in which patients with RAS/BRAF wild-type (n=31), HER2-amplified mCRC and refractory to standard treatments (chemorefractory) were enrolled. Diagnostic algorithms similar to that of HERACLES A were also used. At data cut-off, the ORR was 9.7%, the mPFS was 4.1 months and disease control rate 77.4%. Although, HERACLES-B trial did not reach its primary end point of ORR, low toxicity as well as high disease control rate support its therapeutic potential [155].

In addition, in MyPathway trial, which is a multiple basket, open-label, phase IIA study, pertuzumab in combination with trastuzumab was assessed in patients with HER2-amplified chemorefractory mCRC. ORR was 32%, mPFS 2.9 months and mOS 11.5 months. This trial confirmed the crucial role of HER2-targeted treatment with pertuzumab/trastuzumab and a chemotherapy-free regimen in patients with HER2-positive mCRC as well as the importance of molecular testing in colorectal cancer [156].

Two other studies also confirmed the role of anti-HER2 targeting in the management of mCRC. Firstly, MOUNTAINEER was a phase II trial in which tucatinib and trastuzumab was studied in patients with chemorefractory RAS-WT disease. Interim analysis showed a ORR 52.2%, mPFS 8.1 months and mOS 18.7 months [157]. In addition, DESTINY-CRC01 was a phase II trial in which the safety and antitumour activity of trastuzumab deruxtecan was investigated [158]. Patients with RAS and BRAF wild-type tumors and disease progression on two or more prior regimens (n=78) were enrolled into three cohorts according to the HER2 expression level (A, B, C classification with the assistance of IHC, ISH). The ORR in cohort A was 45.3%, (43.8% in patients who had previously received HER2-targeted therapy), DCR was 83%, mPFS 6.9 months while mOS wasn't reached. In addition, no responses were observed in cohorts B and C. This study showed that trastuzumab deruxtecan has a durable activity in HER2-positive mCRC refractory compared to standard treatments with a safe profile [158].

NTRK fusions in metastatic colorectal cancer

It is known that *NTRK1*, *NTRK2* and *NTRK3* genes encode the family of tropomyosin receptor kinases (TRK) TRKA, TRKB, TRKC, which are important for the neural system's normal development. When a nerve growth factor (NGF) is attached to a TRK protein, then the latter gets dimerized, phosphorylated and it activates the PI3K, RAS/MAPK/ERK and PLC-gamma signaling cascades. Alterations of the *NTRK* genes have been detected in many types of adult and children's solid tumors. Some examples of malignancies, in which *NTRK* gene rearrangements have been identified are thyroid [159], gliomas [160], lung [161] and colon [162] tumors.

Although the prevalence of *NTRK* gene alteration in colorectal cancer is below 1% - actually it is estimated that the incidence is between 0.23-0.97%- it is really important to identify these patients [163]. The detection of *NTRK* gene fusions can be identified by Next Generation Sequencing (NGS) with the use of RNA or DNA samples of the patient or by FISH. In 1986 the "*TPM3-TRK*" oncogene was found in a patient with colorectal cancer as a result of an intrachromosomal

rearrangement at 1q22-23. That leads to the fusion of the tropomyosin 3 gene (*TPM3*) with a sequence that encodes both transmembrane and intracellular parts of TRK receptor [164].

Larotrectinib was the first TRK inhibitor approved by FDA in November 2018 in the USA. Alexander Drilon and his team demonstrated through their clinical trial that Larotrectinib is efficient in adult and pediatric solid malignancies with *NTRK* fusions. In this clinical trial, 55 patients with *NTRK* positive tumors were treated with Larotrectinib, which is an orally administered small molecule that shows high affinity to the TRK receptor, without affecting other types of kinases. The ORR in this trial was 80% according to investigator's assessment and 75% according to central assessment. 13% of the patients had complete response (CR), while 62% showed partial response (PR). There were 4 patients with metastatic colorectal cancer and three of them responded to the treatment: two showed partial response and the other's disease remained stable. The median period of response was estimated to be 1.8 months. However, there were patients with primary resistance to Larotrectinib, as well as patients who had progressive disease and, as a result, new mutations that produced Larotrectinib resistance appeared. More specifically, these mutations were in the front position (NTRK1 G595R or NTRK3 G623R;), gatekeeper position (NTRK1 F589L;) and the xDFG position (NTRK1 G667S or NTRK3 G696A;) [165].

Entrectinib is also a 1st generation TRK inhibitor, orally administered, but unlike Larotrectinib that targets exclusively the TRK receptor, it is a pan-kinase inhibitor. That means that it also targets ALK and ROS1 proteins [166]. According to clinical trials, Entrectinib was proven to be well tolerated, while it caused clinically important response in patients with solid tumors characterized by *NTRK* positive fusion. Entrectinib showed efficacy in patients independently of whether they had central nervous system metastasis or not. Blinded Independent Central Review (BICR) showed 57.4% ORR and at the same time, 7.4% was CR. The median period of response was estimated to be 10.4 months [167].

Loxo-195 and Repotrectinb constitute the 2nd generation of TRK inhibitors and they were designed to target these mutations that are resistant to Larotrectinib and Entrectinib. Loxo-195 is selective to all three TRK kinases, their alterations and the acquired resistance mutations not only at preclinical level, but also in patients. A promising new therapy for patients with *NTRK* mutation starts with larotrectinib and is followed by LOXO-195 after the acquired resistance mutations appear. The target is to prolong the time period, during which the disease is under control [168].

CONCLUSIONS

During recent years, there is a plateau regarding the advances in the treatment of metastatic CRC compared to the advances in other solid tumors. Obviously, a better understanding of the underlying molecular mechanisms will lead to better characterization and exploitation of emerging new targets thus improving the management and treatment of CRC.

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