Review

From Molecular Biology to Clinical Trials: Toward Personalized Colorectal Cancer Therapy

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Abstract

During the past years, molecular studies through high-throughput technologies have led to the confirmation of critical alterations in colorectal cancer (CRC) and the discovery of some new ones, including mutations, DNA methylations, and structural chromosomal changes. These genomic alterations might act in concert to dysregulate specific signaling pathways that normally exert their functions on critical cell phenotypes, including the regulation of cellular metabolism, proliferation, differentiation, and survival. Targeted therapy against key components of altered signaling pathways has allowed an improvement in CRC treatment. However, a significant percentage of patients with CRC and metastatic CRC will not benefit from these targeted therapies and will be restricted to systemic chemotherapy. Mechanisms of resistance have been associated with specific gene alterations. To fully understand the nature and significance of the genetic and epigenetic defects in CRC that might favor a tumor evading a given therapy, much work remains. Therefore, a dynamic link between basic molecular research and preclinical studies, which ultimately constitute the prelude to standardized therapies, is very important to provide better and more effective treatments against CRC. We present an updated revision of the main molecular features of CRC and their associated therapies currently under study in clinical trials. Moreover, we performed an unsupervised classification of CRC clinical trials with the aim of obtaining an overview of the future perspectives of preclinical studies.

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Introduction

Colorectal cancer (CRC) is a molecular heterogeneous disease.[1] Several genomic alterations have been discovered in the past few years with the technological advances in the development of sequencing platforms. This has led to the integrative characterization of CRC molecular features, such as mutations, promoter methylation, and mRNA expression, which eventually uncovered several pathways important for CRC initiation and progression.[2-4]

The common affected pathways include Wnt signaling, receptor tyrosine kinase (RTK) signaling—with vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), and MET as the main members—phosphoinositide 3-kinase (PI3K), transforming growth factor (TGF)-β, p53, and apoptotic signaling. Targeted therapy against the key components of these signaling pathways has allowed improvements in CRC treatment. However, a significant percentage of patients with advanced CRC will not benefit from these targeted therapies and will be restricted to systemic chemotherapy.

It has been shown that the mechanisms of resistance are directly connected to specific gene alterations, such as *KRAS*-mutated tumors that show resistance to anti-EGFR therapy.[5] Further insights into these mechanisms could enable a deeper understanding of tumor evasion to therapy and might identify specific potential targets that could stratify patients to receive the appropriate treatment. Therefore, a dynamic link between basic molecular research and preclinical studies, which ultimately constitute the prelude to standardized therapies, is very important to provide better and more effective treatments against CRC.

We present an updated revision of the main molecular features of CRC and their associated therapies currently under study in clinical trials. Moreover, we performed an unsupervised hierarchical clustering classification of 352 CRC clinical trials selected from the ClinicalTrials.gov database with the aim of obtaining an overview of the direction-pointing preclinical studies.

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Wnt Pathway

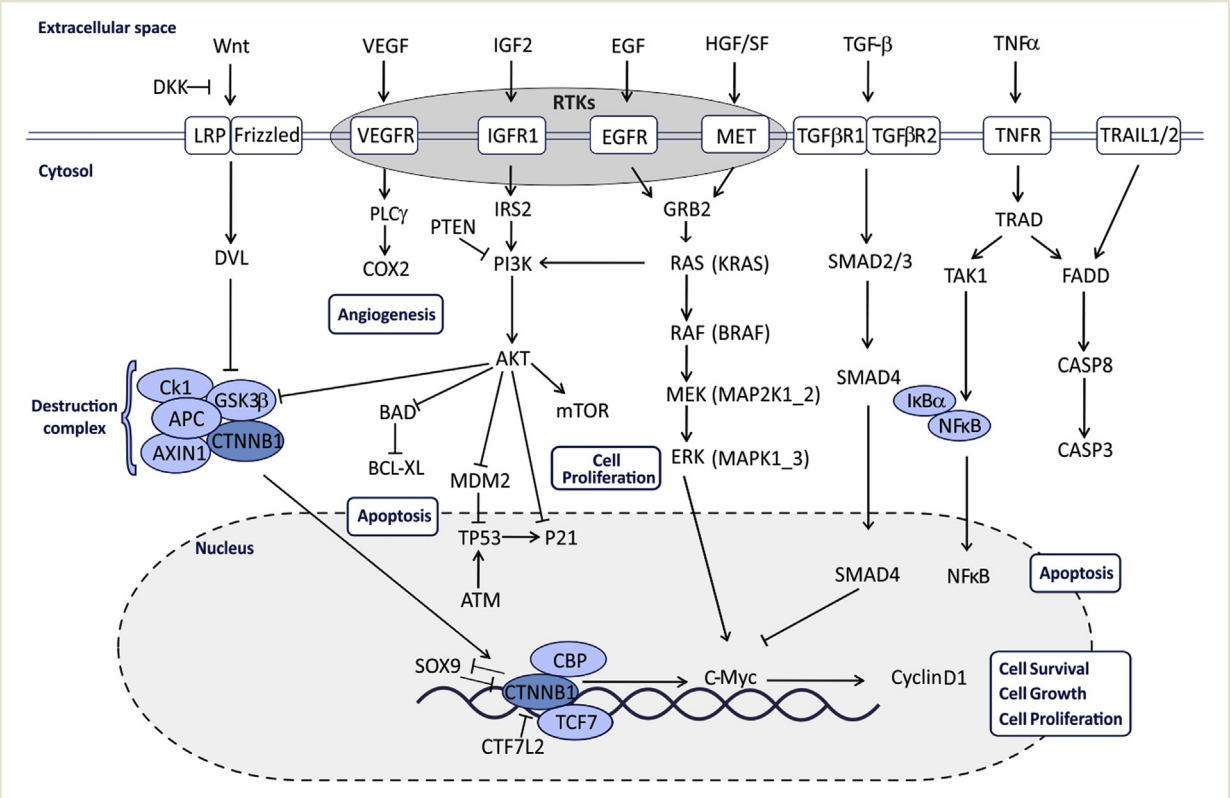
Wnt signaling is a highly conserved pathway involved in developmental processes, such as cell proliferation, differentiation, and polarity.[6] In the canonical Wnt pathway, the tumor suppressor adenomatous polyposis coli, axin, casein kinase 1, and glycogen synthase kinase 3 form the destruction complex that binds to β-catenin (CTNNB1), which is phosphorylated by glycogen synthase kinase 3 and subsequently ubiquitinated by being destroyed in the proteasome.[7] In contrast, when Wnt ligands bind to the lipoprotein receptor-related protein and the frizzled receptor, the cytosolic disheveled protein is activated, and it inhibits CTNNB1 phosphorylation and its consequent degradation. Thus, the protein accumulates in the cytosol and eventually translocates to the nucleus, where it binds to T-cell–specific transcription factor 7, and both participate in the activation of downstream target genes, such as *cMYC,* promoting cell proliferation (Figure 1).

Several components of Wnt signaling might contribute to tumorigenesis when they have been altered genomically.[8] According to the molecular characterization of CRC by The Cancer Genome Atlas group, 94% of the analyzed tumors showed the Wnt pathway was affected,[2] predominately (80%) with inactivating mutations of adenomatous polyposis coli and activating mutations of *CTNNB1*. They also found mutations in *SOX9*, mutations and deletions in T-cell–specific transcription factor 7-like 2 and DKK members (inhibitors of Wnt signaling), and overexpression of the frizzled receptor. Because these alterations could confer an advantage phenotype to malignant cells, targeting Wnt signaling has become one of the main focuses in the development of new targeted therapies for CRC.

Although no drugs targeting the Wnt pathway in CRC have yet been approved by the Food and Drug Administration (FDA), numerous small molecule inhibitors of this pathway have been developed and have been extensively reviewed by Song et al[9] in 2015. Some of them are currently being evaluated in clinical trials.

Because the vast majority of patients with CRC have the Wnt pathway affected in ≥ 1 components, hampering the stratification of patients into those with a good or bad response to therapy, one important challenge is to find the target that minimizes the side effects. A proposed strategy has been the development of inhibitors against molecules that do not constitute the central core of the pathway.[10,11] Inhibition of the interaction between CTNNB1 and Creb-binding protein by the small molecule ICG001, for instance, has shown a decrease in adenoma formation in mouse models of colon cancer.[12] This and similar agents have been considered in preclinical studies.[13-15] In a phase II clinical trial, the Creb-binding protein/CTNNB1 antagonist PRI-724 has been proposed for

Figure 1 Schematic Representation of the Main Pathways Affected in Colorectal Cancer. The Gene Symbols of the RAS-RAF-MEK-extracellular Signal-regulated Kinase (ERK) Signaling Cascade Are Indicated in Brackets



Abbreviations: APC = adenomatous polyposis coli; COX2 = cyclooxygenase 2; DVL = disheveled protein; EGF = epidermal growth factor; HGF = hepatocyte growth factor; IGF2 = insulin-like growth factor-2; LRP = lipoprotein receptor-related protein; TGF-β = transforming growth factor-β; TNF-α = tumor necrosis factor-α; TNFR = TNF receptor; TRAIL = TNF-related apoptosis-inducing ligand; VEGF = vascular endothelial growth factor; VEGFR = VEGF receptor.

testing with chemotherapy and bevacizumab (an anti-VEGF monoclonal antibody) in patients with newly diagnosed metastatic CRC (mCRC) (ClinicalTrials.gov identifier, NCT02413853). In addition, the porcupine inhibitor WNT974 will be tested alone in a phase I study and in a triple combination with LGX818 and cetuximab (an anti-EGFR monoclonal antibody) in a phase II trial for the treatment of patients with mCRC and BRAF and Wnt pathway mutations (ClinicalTrials.gov identifier, NCT02278133). The results of these studies will be relevant to determining the actual efficiency of drugs directed against noncore components of the Wnt pathway in CRC.

Pathways Associated With RTKs

An important group of signaling pathways involved in CRC is the group triggered by RTKs. These include the VEGFR, IGFR1, PI3K, EGFR, and MET pathways.

VEGF Receptors

VEGFs normally bind to their receptors (VEGFRs), promoting angiogenesis (Figure 1). VEGF upregulation has been associated with CRC angiogenesis and survival.[16-20] Consequently, several compounds have been developed against VEGFs or their receptors as therapeutic approaches. To date, 4 drugs have been approved by the National Cancer Institute (NCI). These are the antibodies bevacizumab, directed against VEGF, preventing its receptor binding; ramucirumab, which bind to, and inhibits VEGFR 2; aflibercept, which binds to proangiogenic VEGFs; and the multikinase inhibitor regorafenib, which binds to and inhibits VEGFRs 2 and 3.

Bevacizumab is the only anti-angiogenic agent available for first-line therapy for mCRC. Ramucirumab and aflibercept have been approved for use with FOLFIRI (folinic acid, 5-fluorouracil, irinotecan hydrochloride): aflibercept as second-line treatment and ramucirumab for patients with progression during therapy with FOLFOX (folinic acid, 5-fluorouracil, oxaliplatin) and bevacizumab.[21] Overall, studies have demonstrated that antibody therapy against neoangiogenesis combined with chemotherapy has significantly increased the overall survival (OS) and progression-free survival of patients with mCRC.[22-24] However, multiple and variable-grade side effects have been documented with the use of these antibodies and should be taken into account during the course of treatment.[25-27] Regorafenib, moreover, is the first small molecule inhibitor of RTK that has demonstrated OS benefits in patients with mCRC experiencing progression during standard therapies.[28] Currently, numerous clinical trials are being conducted, using the agent alone or combined with other drugs (ClinicalTrials.gov identifiers, NCT02175654, NCT02199223, NCT02116894, NCT01189903).

Cyclooxygenase 2, induced by hypoxic stimuli and involved in the process of angiogenesis, is overexpressed in CRC and promotes colon carcinogenesis.[29] The use of nonsteroidal anti-inflammatory drugs in preclinical studies has been shown to significantly reduce the risk of CRC.[30] The nonsteroidal anti-inflammatory drugs used in CRC prevention and treatment include aspirin, etodolac, rofecoxib, indomethacin, and celecoxib, among others. Although none of them has yet been approved by the FDA, clinical trials conducted of these drugs have mainly focused on the prevention of the disease, the effects of the drugs on the recurrence of CRC, or the adjuvant treatment of patients with early stages of CRC (ClinicalTrials.gov identifiers, NCT02394769, NCT00888797, NCT00031863).

IGF1R

The expression of IGF1R is associated with malignant transformation and has been found to be overexpressed in CRC.[31] Activation of the IGF1R pathway by ligands such as IGF2 leads to downstream activation of important cellular intermediaries, which, in turn, connect with other pathways and eventually lead to cell growth and proliferation (Figure 1).

Monoclonal antibodies directed against the external cell surface domain of IGF1R interfere with IGF2 binding, thereby preventing IGF1R signaling. Cixutumumab, dalotuzumab, and ganitumab have been used in phase I and II trials, in combination with the anti-EGFR antibodies cetuximab and/or panitumumab in patients with previously treated mCRC.[32-34] Although the results showed an improvement in patient survival, additional clinical trials are needed to validate the utility of these antibodies and evaluate their side effects.

PI3K-AKT-mTOR

The PI3K/AKT is a relevant signaling cascade downstream of both IGFR1 and EGFR and has been involved in colorectal carcinogenesis.[35,36] When PI3K is normally activated by RTK, AKT is recruited and activated by its phosphorylation by PI3K, PDK1, and MTORC2. The activation of AKT leads to inhibition of the pro-apoptotic BCL2 family of proteins, increased degradation of the pro-apoptotic p53 protein by MDM2, increased expression of anti-apoptotic genes, and activation of mTOR proteins, which, in turn, increase protein synthesis.[37,38] All these processes lead to cell growth, survival, and proliferation (Figure 1).

Activating mutations of *PI3KCA* are commonly found in the advanced stages of CRC, predominately in exons 9 and 20; however, little correlation has been found between these mutations and overexpression of PI3K, suggesting that the protein increase might also be attributable to other mechanisms.[2,39] The downregulator of PI3K is PTEN, frequently inactivated by mutations in CRC.[2,37] Overexpression of AKT is also often found in the early stages of CRC.[2,40] Thus, PI3K-AKT signaling has been considered a potential therapeutic target.

Buparlisib, also known as BKM120, is an oral compound that specifically inhibits class I PI3K in the PI3K/AKT signaling pathway. It has been demonstrated to be a safe and well-tolerated drug.[41] The clinical trials NCT01591421 and NCT01304602 are currently underway, recruiting patients with mCRC to be treated with BKM120 combined with panitumumab and irinotecan, respectively. Both studies are in phase I; thus, it is too early to estimate the effectiveness of buparlisib in future therapies.

BYL719 is another PI3K inhibitor of the PI3K/AKT signaling pathway under evaluation. Currently, 1 clinical trial (ClinicalTrials.gov identifier, NCT01719380) has been proposed to assess the safety and efficacy of BYL719 when combined with cetuximab and LGX818 in patients with *BRAF*-mutant mCRC. LGX818 is a drug originally developed for the treatment of melanoma with the *BRAF V600E* mutation, which is the most frequent *BRAF* mutation in CRC. Because it has been demonstrated that *BRAF*-mutated CRC

is resistant to anti-EGFR therapies,[42] the combination of cetuximab and LGX818 in the first phase and the ulterior inhibition of PI3K signaling in the second phase is a promising strategy for the treatment of this group of patients.

The compound MK-2206 binds to and inhibits the activity of AKT isoforms. This drug was previously tested in solid tumors in phase I trials and resulted in tolerable toxicity and effective AKT-signaling blockade.[43] Very recently, the drug was evaluated in patients with advanced CRC in combination with selumetinib, an inhibitor of mitogen-activated protein kinase (MEK or MAPK/extracellular signal-regulated kinase) 1 and 2.[44] However, no responses were observed, and the level of target inhibition, pre-established at 70%, was not achieved. In contrast, 1 study is underway that proposes the use of MK-2206 as monotherapy in patients with *KRAS* wild-type and *PI3KCA*-mutated mCRC (ClinicalTrials.gov identifier, NCT01186705). The results obtained will be important to establishing whether MK-2206 is more effective given alone or in combination with other drugs.

The other cross-talk downstream RTK is AKT-mTOR (Figure 1). AKT induces full inhibition of the negative regulator of mTOR, TSC2, and activation of mTOR, both through direct phosphorylation and by inhibition of phosphorylation of AMP-activated protein kinase, which is an activator of TSC2.[45] Several clinical trials have been completed of mTOR inhibitors such as everolimus or temsirolimus, which are analogs of rapamycin. Recently, Hecht et al[46] determined the feasible doses of everolimus to be administered with irinotecan and cetuximab as second-line therapy for mCRC. Other trials have evaluated the use of everolimus alone (ClinicalTrials.gov identifier, NCT00419159) or combined with the antibody bevacizumab (ClinicalTrials.gov identifier, NCT00597506) or panitumumab (ClinicalTrials.gov identifier, NCT01139138; currently recruiting). However, the results have not yet been published. Regarding temsirolimus, although it has been shown to decrease cellular resistance to cetuximab in colon cancer cells and the corresponding clinical trials have been conducted, to date, no results have been proposed.[47] Sirolimus is another inhibitor of mTOR currently being evaluated to be administered before and during radiotherapy to treat rectal cancer (ClinicalTrials.gov identifier, NCT00409994). In summary, the use of mTOR inhibitors in the treatment of CRC is still under careful investigation.

EGFR

The FDA-approved antibodies cetuximab and panitumumab are widely used in therapy and have been shown to be highly effective in a subset of patients with mCRC. Lièvre et al[48] and Allegra et al[49] have demonstrated, however, that when the EGFR downstream effector *KRAS* is mutated, the tumor shows resistance to EGFR antibodies. Since then, the *KRAS* mutation has been considered the beginning of personalized therapy in patients with mCRC.[50] More than 35% of CRC cases have the *KRAS* gene mutated, predominately (80%-90%) in codons 12 or 13, leading to inhibition of guanosine triphosphatase activity.[51] These data are now being considered to stratify patients—profiled for *KRAS* mutations—who are going to receive cetuximab or panitumumab.

The other problem with targeting EGFR is that 65% of *KRAS* wild-type CRC cases also have no response to anti-EGFR therapy.[52] It has been postulated that several genetic or epigenetic causes could explain the resistance to anti-EGFR therapy. These include mutations in the *BRAF* and *PIK3CA* genes, methylation and silencing of the *EGFR*, increased expression of other RTKs such as IGFR1 and MET, and upregulation of the MET ligand hepatocyte growth factor, among others.[50,53] These biologic features have encouraged researchers to develop new alternative therapies using multiple agents simultaneously.[54]

The *BRAF* gene will be mutated in about 10% of CRC cases and has been associated with a poor prognosis.[55,56] It has been demonstrated that *BRAF* and *KRAS* mutations are mutually exclusive, with the latter more predominant in hypermutated tumors of the right colon, with a methylator phenotype (CpG island methylator phenotype) and instability of microsatellites. In contrast, *BRAF* mutation is commonly found in association with microsatellite stable colorectal tumors, irrespective of their location.[2,50] In addition to these contrasting differences, both *BRAF*- and *KRAS*-mutant tumors have shown resistance to anti-EGFR therapies. Furthermore, it was recently demonstrated in a meta-analysis study that cetuximab and panitumumab therapy in patients with advanced CRC who have *RAS* wild-type and *BRAF*-mutant status did not benefit from treatment. The investigators suggested that before undergoing anti-EGFR treatment, patients should be assessed for *BRAF* mutations, in addition to *KRAS* status.[57]

Activating the *BRAF V600E* mutation leads to phosphorylation and activation of the downstream MEK1 and MEK2 kinases (MAP2K1/2). Specific inhibitors of BRAF have been developed for patients bearing *BRAF* mutations. In this sense, vemurafenib and dabrafenib are being studied in clinical trials. The use of vemurafenib combined with panitumumab in patients with mCRC has been shown to be well tolerated, with significant disease control.[58] In addition, 1 phase II clinical trial is underway assessing the combination of cetuximab and irinotecan with or without vemurafenib (ClinicalTrials.gov identifier, NCT02164916), but no results have yet been obtained. Furthermore, dabrafenib is being evaluated in a multitarget study combined with panitumumab and the MAP2K1/2 inhibitor trametinib (ClinicalTrials.gov identifier, NCT01750918). Previously, a clinical trial of combined BRAF/MAP2K inhibition with dabrafenib and trametinib in patients with the *BRAF V600E* mutation had reported a response rate of 12%.[59] Overall, vemurafenib and dabrafenib have not yet been approved by the FDA.

In an in vitro model, Ahronian et al[60] found that acquired resistance to the BRAF/EGFR or BRAF/MEK inhibitor combinations in *BRAF*-mutant CRC cell lines is associated with activating *KRAS* mutations in exon 2 through sustained MAPK pathway activation. Other alterations, such as *KRAS* amplification, *BRAF* amplification, and *MEK1* mutation, were also identified in the resistance to combinations of BRAF/EGFR or BRAF/MEK inhibitors and sustained MAPK activation.[60] These results are relevant to the stratification of patients to future therapies according to these genomic changes, thus avoiding exposure to treatment resistance with the consequential side effects.

Pimasertib, selumetinib, and trametinib are the specific MEK1/2 inhibitors currently used in clinical trials. Although some of these trials have been completed, even more studies are needed to draw conclusions about the effectiveness of these drugs in patients with *BRAF*-mutant CRC.[44,61]

MET

MET signaling shares multiple downstream pathways with EGFR, including the Ras-Raf-MAPK and PI3K/AKT pathways (Figure 1). Both the receptor (cMET) and its ligand (hepatocyte growth factor) have been found to be overexpressed in CRC and linked to a poor prognosis.[62-64] Several preclinical approaches have been tried in an attempt to inhibit these targets. Onartuzumab and rilotumumab are monoclonal antibodies developed against the extracellular domain of cMET and the hepatocyte growth factor, respectively. Van Cutsem et al[33] recently demonstrated for the first time that combining rilotumumab with panitumumab in patients with previously treated mCRC with wild-type *KRAS* is significantly beneficial in the objective response rate, suggesting the need to stratify patients who could benefit from combined therapy with antibodies directed against the EGFR and MET pathways. The use of onartuzumab is under phase II study in combination with bevacizumab (ClinicalTrials.gov identifier, NCT01418222).[65] Another inhibitor of MET, called tivantinib, binds to the c-Met protein and disrupts the MET signal transduction pathway. A clinical trial is underway of tivantinib combined with cetuximab and irinotecan for the treatment of patients with mCRC (ClinicalTrials.gov identifier, NCT01075048). Which of these approaches causes the minimal side effects, whether the use of biologic compounds such as the aforementioned antibodies or the chemical inhibitor tivantinib, has yet to be determined.

TGF-β Pathway

Inactivation of TGF-β signaling in CRC also contributes to carcinogenesis. The increased proliferation results from the loss of the growth inhibition mediated by TGF-β (Figure 1). The most frequent mechanism producing inactivation of pathway signaling is the mutation of *TGFBR2*.[66] According to The Cancer Genome Atlas, *TGFBR2* was found to be mutated in 62% of the hypermutated group of CRC, with this genomic alteration more frequent in tumors with microsatellite instability, *BRAF V600E* mutation, a methylator phenotype, and a right colon location.[2] Furthermore, it has been recently shown that the micro-RNA miR-135b acts as another factor contributing to the downregulation of TGFBR2 expression and, consequently, an increase in cell proliferation.[67] miR-135b has been found to be frequently deregulated in CRC; therefore, it has been proposed as a valuable biomarker and target for therapy.[68] The genes encoding the downstream proteins of TGF-β signaling are also affected in CRC, such as the loss of the genes *SMAD2* and *SMAD4* caused by the deletion of chromosome 18q.[69] Downregulation of *SMAD4* has been shown to be predictive of a poor response to chemotherapy and significantly shorter survival compared with the response and survival of patients with tumors expressing high levels of *SMAD4*.[70] The identification of this and the different genomic alterations of TGF-β signaling could help to identify those patients at risk of developing cancer or who could have CRC resistant to standard therapies. More clinical trials regarding this topic are needed.

Tumor Necrosis Factor-α Pathway

Different clinical and experimental studies have demonstrated that tumor necrosis factor (TNF)-α plays an important role in CRC progression.[71,72] TNF-α is a cytokine associated with many functions related to the early immune response. Among others, TNF-α stimulates the acute phase response, mediates the inflammatory response, activates other cytokines, and increases vascular permeability.[74] It has been considered a tumor-inducing factor by promoting angiogenesis and suppressing immune-mediated tumor elimination.[73,74] The compound lenalidomide has been used in clinical trials for the treatment of patients with CRC. This drug inhibits TNF-α production, stimulates T cells, reduces the serum levels of VEGF and basic fibroblast growth factor, and inhibits angiogenesis. However, a phase II study that used lenalidomide combined with cetuximab in patients with mCRC showed no clinical benefits for the use of this drug.[75] Since then, no reports have been published about its effectiveness against CRC. Alternative available drugs targeting TNF-α should be studied.

Apoptotic Pathways

Finally, instead of inhibition of the pathways associated with cell growth and proliferation, increasing evidence has led to the development of therapeutic strategies to activate the pathways linked to apoptosis.[76] The TNF-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent. The recombinant human protein TRAIL, dulanermin, has been evaluated in clinical trials; it binds to and activates TRAIL receptors 1 and 2, which might activate caspases and induce p53-independent apoptosis in TRAIL receptor 1- or 2-expressing tumor cells (Figure 1). Two clinical trials have already been completed of dulanermin combined with standard chemotherapy.[77,78] The study by Wainberg et al[77] demonstrated that dulanermin is well tolerated in patients with advanced CRC. In addition, Lim et al,[78] who evaluated dulanermin in a patient with BRAF-mutant mCRC, emphasized that the patient continued receiving FOLFIRI plus dulanermin therapy for a period that exceeded the known median OS for patients with BRAF-mutant malignancy treated under the same conditions without dulanermin. Despite this observation, the investigators could not ascertain whether the observed effect had resulted from dulanermin or random. Therefore, additional clinical trials recruiting patients with BRAF-mutant tumors should be performed to establish whether targeting the TRIAL pathway with this drug will be significantly beneficial.

Epigenetic Therapy

DNA methylation (silencing gene expression), histone alterations, chromatin remodelers, and variations in noncoding RNAs such as micro-RNAs are common epigenetic features during CRC progression.[79] These alterations can be reversed by DNA demethylating and histone acetylating agents. Thus, targeting epigenetic changes offers new perspectives in the treatment of CRC. DNA methyltransferase inhibitors and histone deacetylase inhibitors have shown promising results in controlling cancer progression.[80] Although it is in an early stage of research, different epigenetic inhibitors are being evaluated in combination with traditional therapies in patients with advanced CRC. The safety and tolerability of the DNA methyltransferase inhibitor SGI-110, given together with irinotecan hydrochloride or regorafenib alone, will be assessed in patients with mCRC (ClinicalTrials.gov identifier, NCT01896856). Similarly, vorinostat, which was the first histone deacetylase inhibitor used in clinical trials in combination with

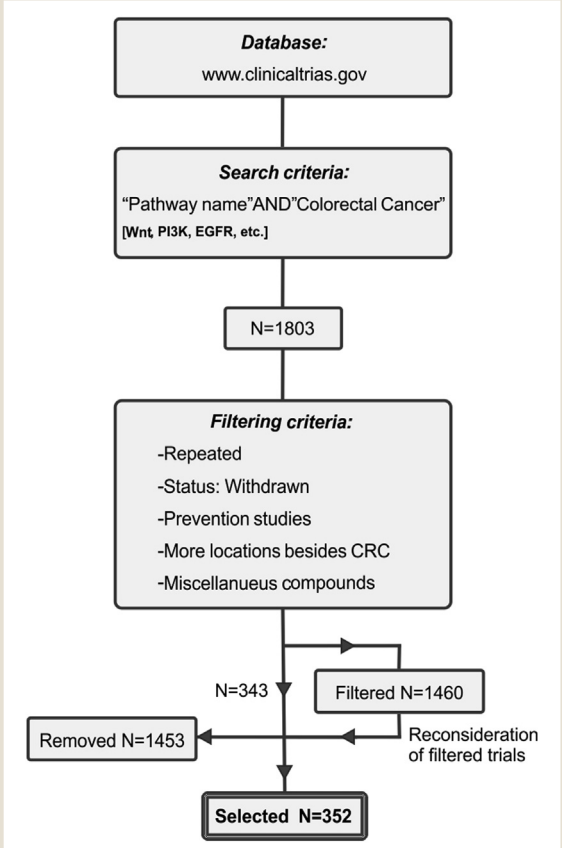
other drugs to treat CRC patients, but without published results, will be tested in patients with chemotherapy-refractory mCRC (ClinicalTrials.gov identifier, NCT02316340). Overall, epigenetic treatment in CRC is in its experimental stage but is expected to constitute an important tool to support traditional therapies.

The major genetic alterations in CRC and their targeted therapies in clinical trials are listed in Supplemental Table 1 (available online).

Overview of CRC Clinical Trials

With the aim of obtaining the overall status of clinical trials of CRC, a searching workflow was conducted, finding a total of 352 clinical trials from the ClinicalTrials.gov database (Figure 2; Supplemental Table 2; available online). These studies involved a total of 110 drugs (Supplemental Table 3; available online).

Figure 2 **Methodologic Workflow for Colorectal Cancer (CRC) Clinical Trial Selection. Using the Filtering Criteria, Trials That Were Repeated Through Different Search Approaches, Trials Whose Status Was Withdrawn, Trials of Only Prevention, Trials in Which Locations Other than CRC Were Considered for Recruitment, and Trials That Evaluated Atypical Compounds in CRC Were Excluded. In a Second Revision of the Excluded Trials, 9 Were Reincorporated, Some of Which Were Prevention Studies. Although They Had Not Passed the Filter Criteria, They Had Studied Drugs That Could Be Relevant to the Posterior Analysis**



To enable unsupervised classification of these trials, we used the Multi Experiment Viewer software.[81] First, a binary matrix (presence, absence) of the studies (columns) versus drugs (rows) was built. The interception between each row and each column was filled with 1 or 0 according to the presence or absence, respectively, of the drug in the study. The matrix was then loaded into the Multi Experiment Viewer, and the hierarchical clustering method was applied, according to the Euclidean distance metric and the complete linkage clustering method.[82] A heat map was obtained, in which each row represented a specific drug and each column represented each study (Figure 3A). The dark blue boxes represent the presence of the drug in the respective study. A dendrogram tree above the columns grouped the studies into clusters. Using a divisive strategy through a top down approach, hierarchical clustering classified the clinical trials into 3 main clusters (clusters 1, 2, and 3), which, in turn, were subdivided into 2, 3, and 2 groups, respectively (Figure 3A, Supplemental Table 4; available online). Cluster 1 preferentially grouped those studies in which systemic chemotherapy with the traditional drugs, 5-fluorouracil, leucovorin, and oxaliplatin, was tested. Cluster 2 included the studies that had involved a combination of chemotherapy and targeted therapy, with bevacizumab as the main agent. Finally, cluster 3, in contrast to cluster 1, mainly included studies that had focused on targeted therapy, with anti-EGFR antibodies as the main agents. To better understand the nature of this classification, the clusters are characterized in detail and discussed in the following sections.

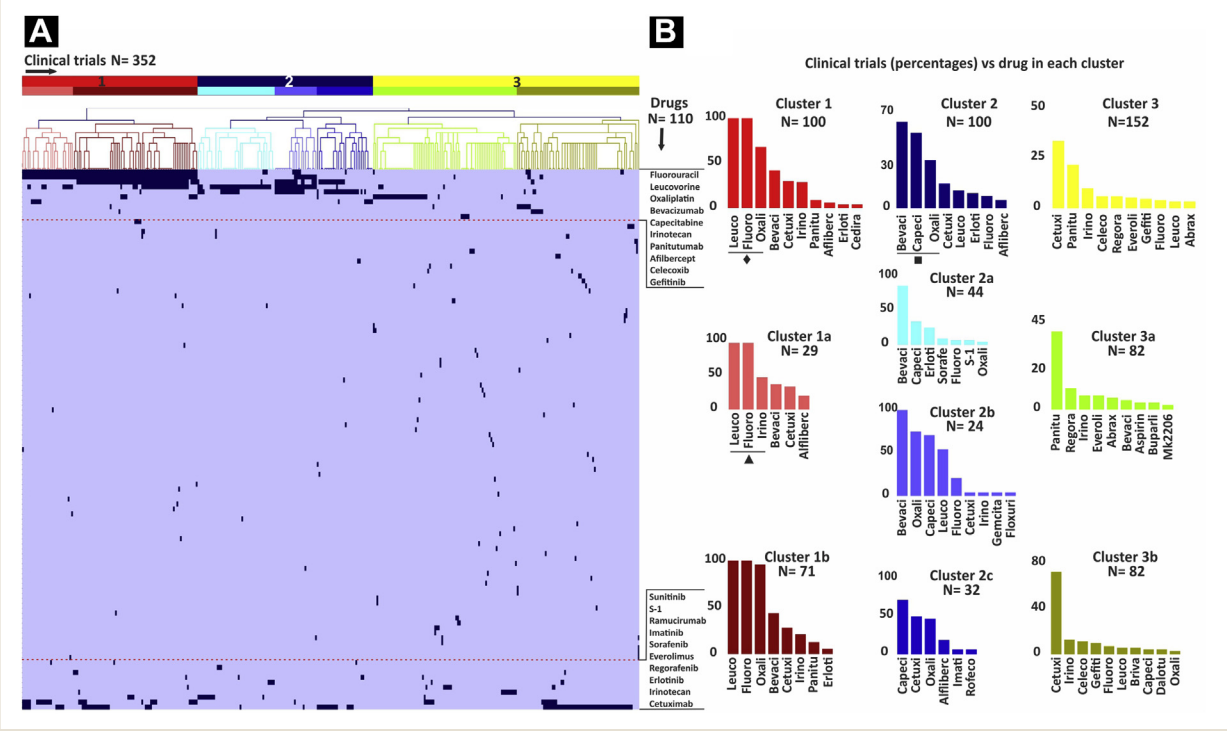
Cluster 1

Cluster 1 grouped 100 of 352 clinical trials (28%). Only the drugs present in $>$ 4% of the clinical trials (Figure 3B) were considered. Interestingly, 100% of these trials included in their protocols 5-fluorouracil and leucovorin as treatment agents, independently of any other drug. Also, 68% of the studies included oxaliplatin. The combination of these 3 drugs constitutes the FOLFOX regimen of standard chemotherapy. Moreover, irinotecan, the other drug included in standard regimens, was present in 30% of the studies. FOLFOX plus irinotecan constitutes FOLFOXIRI. Of the biologic compounds, 40% of the trials used the anti-VEGF bevacizumab, 30% the anti-EGFR cetuximab, 9% the anti-EGFR panitumumab, 6% the anti-VEGF ziv-aflibercept, and 4% everolimus, an inhibitor of the mTOR pathway, and cediranib, which blocks VEGF signaling. Thus, although 100% of the studies in cluster 1 investigated the use of standard chemotherapy, $\geq$ 40% also included targeted therapy, which was mainly divided into anti-VEGF and anti-EGFR targets.

Cluster 1 segregated into clusters 1a and 1b (Figure 3). In cluster 1a were those trials that used FOLFIRI (30%) and the antibodies bevacizumab, cetuximab, and aflibercept, predominately. In contrast, cluster 2a preferentially grouped studies that included the FOLFOX regimen and a low percentage of targeted therapies.

Other relevant characteristics of the trials were also evaluated, such as the regimen drug (1 drug vs. $\geq$ 2 drugs), trial status, organ location, and other conditions of recruitment (Table 1). As expected, 100% of the trials from cluster 1 used $>$ 1 drug, 52% of the studies (the same percentage of the total studies) had been completed, 41% are underway with differing recruitment status, and only 7% had been terminated for different reasons. Also, 72%

Figure 3 Hierarchical Clustering Classification of Colorectal Cancer (CRC) Clinical Trials. (A) Heat Map of 352 Studies (Columns) Versus Drugs Proposed to Be Used in Them (Rows, n = 110). Three Main Clusters, Indicated in Upper Bars, Were Observed: Cluster 1 (red), Cluster 2 (Dark Blue), and Cluster 3 (Yellow). According to the Dendrogram Branching, Cluster 1 Was Subdivided Into Cluster 1a (Pink) and 1b (Burgundy); Cluster 2 Was Subdivided Into Cluster 2a (Light Blue), 2b (Lilac), and 2c (Blue); and Cluster 3 Was Subdivided Into 3a (Light Green) and 3b (Military Green). The Most Significant Drugs Involved in the Classification Are Highlighted to the Right of the Heat Map. (B) Bar Charts of the Percentage of Studies Versus the Drugs for Each Cluster. Diamonds Indicate Drugs of FOLFOX (Folinic Acid, 5-Fluorouracil, Oxaliplatin) Regimen; Triangles, Drugs of FOLFIRI (Folinic Acid, 5-Fluorouracil, Irinotecan Hydrochloride) Regimen; and Squares, Drugs of CAPOX-B (Capecitabine, Oxaliplatin, Bevacizumab) Regimen



of the cluster 1 trials recruited mCRC patients, 8 points greater than the percentage for all the studies. The other conditions of recruitment varied among the studies and were required in $\geq$ 4% of the trials.

We concluded that cluster 1 mainly included trials that had focused on improvement in the administration of standard chemotherapy for patients with advanced CRC with the help of targeted therapy against the VEGFR or EGFR pathway. Also, except for everolimus and cediranib, which were only involved in 4% of the studies, the main drugs used in the trials in cluster 1 have all been approved by the FDA.

Cluster 2

Similar to cluster 1, 100 clinical trials (28%) were included under cluster 2. The main feature of the trials in cluster 2 was the use of capecitabine (55%) and/or oxaliplatin (35%) as standard chemotherapy and bevacizumab (63%), among others, as targeted therapy (eg, the CAPOX-B regimen). Three subclusters arose from the second branching of the hierarchical clustering for cluster 2.

Cluster 2a included 86% of the trials using bevacizumab, 34% of those using capecitabine, and 25% of those using the EGFR inhibitor erlotinib. Other drugs such as sorafenib and S-1 were also investigated in the studies in cluster 2a. Erlotinib, usually tested in combination with bevacizumab or cetuximab and standard chemotherapy for dual pathway inhibition of patients with pretreated mCRC, has been shown to be well tolerated.[83,84] Sorafenib, however, was recently reported to not be effective for the treatment of *KRAS*-mutated mCRC in combination with cetuximab.[85]

One interesting issue of cluster 2a, compared with cluster 1, was that 34% of the trials involved the use of only 1 drug (Table 1), in most cases, bevacizumab or erlotinib, because these were post-chemotherapy studies of patients with mCRC. Previous results have shown, however, that the use of bevacizumab as monotherapy is inferior to both FOLFOX and FOLFOX plus bevacizumab in terms of the response rate and progression-free survival and that the OS duration is longer for patients receiving FOLFOX plus bevacizumab. These findings suggest that multidrug therapy is better than bevacizumab monotherapy per se.[86]

Cluster 2b was segregated by the use of oxaliplatin and capecitabine (CAPOX) combined with bevacizumab. Very recently, the effectiveness of maintenance treatment with capecitabine plus bevacizumab in patients with mCRC previously treated with CAPOX-B was demonstrated.[87] This finding should encourage the increase of clinical trials using the combination of these drugs.

Table 1 Frequency Description of Main Features of CRC Clinical Trials Included

Variable	All (n = 352)	Cluster 1			Cluster 2				Cluster 3		
		1 (n = 100)	1a (n = 29)	1b (n = 71)	2 (n = 100)	2a (n = 44)	2b (n = 29)	2c (n = 32)	3 (n = 152)	3a (n = 82)	3b (n = 70)
Regimen											
≥ 2 Drugs	266 (76)	100 (100)	29 (100)	71 (100)	78 (78)	29 (66)	24 (100)	25 (78)	80 (53)	29 (35)	51 (73)
1 Drug	96 (27)	0 (0)	0 (0)	0 (0)	22 (22)	15 (34)	0 (0)	7 (22)	72 (47)	53 (**65**)	19 (27)
Status											
Active, NR	56 (16)	14 (14)	7 (24)	8 (11)	21 (21)	3 (7)	7 (29)	11 (**34**)	21 (14)	12 (15)	9 (13)
Completed	182 (52)	51 (51)	11 (38)	41 (58)	56 (56)	30 (68)	12 (50)	14 (44)	75 (49)	41 (50)	34 (49)
Recruiting	64 (18)	21 (21)	7 (24)	14 (20)	12 (12)	6 (14)	3 (13)	3 (9)	31 (20)	16 (20)	15 (21)
Not yet recruiting	8 (2)	4 (4)	0 (0)	4 (6)	1 (1)	0 (0)	0 (0)	1 (3)	4 (3)	2 (2)	2 (3)
Terminated	42 (12)	10 (10)	4 (14)	4 (6)	10 (10)	5 (11)	2 (8)	3 (9)	21 (14)	11 (13)	10 (14)
Localization											
CC	6 (2)	4 (4)	0 (0)	4 (6)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1)	1 (1)	1 (1)
RC	25 (7)	4 (4)	0 (0)	4 (6)	12 (12)	5 (11)	1 (4)	6 (**19**)	9 (6)	3 (4)	6 (**9**)
CRC	94 (27)	19 (19)	6 (21)	13 (18)	23 (23)	11 (25)	6 (25)	6 (19)	55 (36)	23 (28)	32 (46)
mCRC	227 (64)	73 (**73**)	23 (**79**)	50 (**70**)	65 (65)	28 (64)	17 (71)	20 (63)	86 (57)	55 (67)	31 (**44**)
Conditions											
Preoperative	14 (4)	3 (3)	0 (0)	2 (3)	6 (6)	2 (**5**)	1 (4)	3 (**9**)	6 (4)	2 (2)	4 (6)
Pretreated	15 (4)	4 (4)	1 (3)	3 (**4**)	3 (3)	1 (2)	0 (0)	2 (6)	8 (5)	3 (4)	5 (7)
Mutated genes	16 (5)	3 (3)	1 (3)	2 (3)	1 (1)	1 (2)	0 (0)	0 (0)	12 (**8**)	7 (**9**)	5 (**7**)
WT genes	35 (10)	10 (10)	2 (7)	6 (8)	0 (0)	0 (0)	0 (0)	0 (0)	25 (**16**)	13 (**16**)	12 (**17**)
Unresectable	13 (4)	5 (5)	1 (3)	4 (6)	6 (6)	3 (7)	1 (4)	2 (6)	2 (1)	1 (1)	1 (1)
Other	32 (9)	7 (7)	1 (3)	7 (10)	9 (9)	5 (11)	0 (0)	4 (13)	16 (11)	9 (11)	7 (10)
ND	127 (36)	68 (68)	23 (79)	47 (66)	75 (75)	32 (73)	22 (92)	21 (66)	83 (55)	47 (57)	36 (51)

Data presented as n (%).
The most relevant percentages in the comparison of the variables of trials, in each cluster, are highlighted in bold.
Abbreviations: ND = not determined; NR = not recruiting; WT = wild type.

A high proportion of trials in cluster 2b are currently active and recruiting patients (Table 1).

The last group of cluster 2 was mainly defined by trials that proposed the use of the anti-EGFR cetuximab combined with capecitabine and/or oxaliplatin. In smaller percentages, trials with aflibercept and the inhibitors imatinib and the withdrawn rofecoxib were also represented. The combination of cetuximab and capecitabine has been shown to benefit elderly patients with advanced CRC.[88] The combination of capecitabine plus oxaliplatin and cetuximab is frequently administered as neoadjuvant therapy for patients with rectal cancer, because cluster 2b included the greatest recruitment of patients with rectal cancer (Table 1).

The use of aflibercept in targeting the angiogenesis of mCRC has yielded controversial results. Although it has shown beneficial activity in patients with mCRC previously treated with FOLFIRI, several toxic side effects have been documented, in addition to no improvement compared with the use of bevacizumab.[26,89] Therefore, it is important to continue evaluating this drug in clinical trials.

In summary, cluster 2 included preclinical studies that mainly focused on the evaluation of targeted therapies against neoangiogenesis with bevacizumab as the principal agent and aflibercept combined with standard chemotherapy. It also included a significant number of preoperative studies of rectal cancer, with capecitabine, oxaliplatin, and cetuximab as the key agents.

Cluster 3

Cluster 3 included 152 trials (44%), and cluster 3 was clearly subdivided into 2 subclusters (Figure 3A). The use of the anti-EGFR antibodies cetuximab (34%) and panitumumab (22%) was dominant in these trials. These 2 antibodies constitute the main force of segregation into the 2 subclusters, followed by irinotecan (10%), celecoxib (6%), regorafenib (6%), and everolimus (5%), among others (Figure 3B). Panitumumab has usually been proposed to be administered alone or in diverse combinations with standard chemotherapy and/or targeted agents. The latter include new agents such as trametinib, an anti-ERBB2 monoclonal antibody; dabrafenib, a BRAF inhibitor; and cabozantinib, a multi-RTK inhibitor, including MET.[90-92] Multitarget therapy is a promising approach for different groups of mCRC patients carefully stratified according to genomic features such as *KRAS*, *BRAF*, and *PI3K* status (wild type or mutated). These conditions of recruitment were the most prevalent in cluster 3 compared with the other clusters (Table 1).

Regorafenib was used in the trials in cluster 3a. This compound is one of the newest approved by the FDA for its use in patients with previously treated advanced mCRC. Clinical trials are now focusing on testing this compound, administered alone or with other targeted agents, in CRC and mCRC. Furthermore, albumin-bound paclitaxel (Abraxane), approved for use in other cancers such as lung and breast cancer, is also being considered for CRC preclinical therapy, according to the studies in cluster 3a.

Although cluster 3b mainly included trials of cetuximab (73%) and the drugs used in standard chemotherapy, similar to panitumumab, several trials in cluster 3b combined the anti-EGFR antibody with a myriad of different drugs, many of which have not been previously used for CRC. These included tivantinib (an inhibitor of the c-MET protein), MM-121 (anti-ERBB3), PKI-587 (an inhibitor of the PI3K/mTOR signaling pathway), neratinib (an inhibitor of the HER-2 receptor tyrosine kinase), sorafenib tosylate (which blocks the enzyme RAF kinase), lenalidomide (which inhibits TNF-α production), and vemurafenib (an inhibitor of BRAF V600E kinase), to name a few.

Knowing the resistance to anti-EGFR therapy shown by some CRC tumors, it is clear that the trend of the trials grouped in cluster 3b was toward targeted and personalized therapy to establish the patient groups that share specific molecular features defining ≥ 1 affected pathways to be targeted with specific drugs from multiple flanks.

Recapitulation of CRC Therapy

Strikingly, the clinical trials classification obtained in the present review has recapitulated the chronology of CRC therapy.[93] Cluster 1 mainly referenced traditional chemotherapy, with the commonly used agents leucovorin, 5-fluoruracil, and oxaliplatin, constituting adjuvant FOLFOX therapy. FOLFOX has become the established cytotoxic regimen for the treatment of mCRC, with an average increase of 2 years in OS. The FOLFOX regimen was first studied in 2004 by André et al.[94] Later in 2004, a crossover study investigated the efficacy of FOLFIRI in patients with mCRC.[95] Souglakos et al[96] then evaluated and compared the efficacy of FOLFIRI with that of FOLFOXIRI. Consistently, cluster 1a included trials of the FOLFIRI regimen and cluster1b, trials of the FOLFOXIRI regimen.

The use of bevacizumab as targeted therapy in mCRC was also reported in 2004, with the evaluation of the antibody with fluorouracil in a phase III trial.[97] However, it was not until 2011 that bevacizumab was investigated in patients with CRC.[98] By 2004, capecitabine was introduced as an improvement in the delivery of 5-fluorouracil.[99] The combination of bevacizumab and capecitabine with oxaliplatin, irrespective of other drugs is the main feature of cluster 2, which included a large number of trials that recruited patients with CRC, instead of patients with mCRC, in contrast to that trials in cluster 1 (Table 1).

The efficacy of cetuximab in the treatment of mCRC patients was first evaluated in the CRYSTAL (Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer) study.[100] Furthermore, the efficacy of panitumumab was evaluated in PRIME (Panitumumab Randomized Trial in Combination With Chemotherapy for Metastatic Colorectal Cancer to Determine Efficacy).[101] In both cases, *KRAS* status was evaluated for patient recruitment. Cluster 3 of our classification was characterized by trials using both anti-EGFR antibodies. In addition, this cluster included the largest number of trials with recruitment requiring evaluation of the status of *KRAS* and *BRAF* genes (Table 1). Cluster 3 also included trials of regorafenib, recently approved by the FDA for treating patients with advanced gastrointestinal stromal tumors, based on the results from Demetri et al.[102] Finally, cluster 3 had a greater proportion of studies evaluating 1 drug as a monotherapy approach compared with clusters 1 and 2. Many of these could be promising new agents.

Thus, our general survey of the preclinical studies of CRC has allowed us to draw the landscape of the clinical trials that ultimately constitute the prelude of future cancer therapies. Our classification of clinical trials established 3 main groups, which, in turn, summarize the evolution of therapy against CRC. The first group

(cluster 1) included a large group of trials aimed at improving traditional systemic treatments combined with targeted therapy in patients with advanced CRC. A second group (cluster 2) included intermediate trials, in which 5-fluorouracil was replaced by capecitabine and anti-VEGF antibodies such as bevacizumab were incorporated. Finally, the last group (cluster 3) focused on the use of targeted therapies (anti-EGFR antibodies), the testing of new drugs, the use of monotherapy, and, fundamentally, the appropriate stratification of patients according to their molecular profile to find patients more likely to have a good response to therapy. This last group describes, in our view, the characteristics of future therapies for CRC.

Conclusion

Increasing evidence has shown that CRC can be considered to include many distinct molecular diseases, characterized by a partially defined pattern of molecular changes that affect various molecular pathways. This diversity of tumors has challenged the therapies developed during the past years, leading to the need to recruit patient groups with similar molecular alterations, which can be addressed with more personalized therapies.

In the present review, we have reported the most salient genomic and transcriptomic features of CRC and the various therapeutic agents directed against these potential targets. We also performed a general survey of the clinical trials associated with CRC and an unsupervised classification that also summarized the evolution of therapies for CRC. From the results of the present analysis, we have concluded that although a trend has been shown in improving traditional therapies, including systemic therapy and VEGF- and EGFR-targeted therapy, an increasing number of clinical trials have focused on the use of new drugs directed against specific pathways to be used alone or in combination. The correct stratification of patients and the appropriate choice of therapeutic agents will eventually lead to significant advances in the treatment of CRC.

Supplemental Data

The supplemental tables accompanying this article can be found in the online version at http://dx.doi.org/10.1016/j.clcc.2015.11.001.

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