Current targeted therapies in the treatment of advanced colorectal cancer: a review

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***Abstract*:** Treatment strategies for metastatic colorectal cancer (mCRC) patients have undergone dramatic changes in the past decade and despite improved patient outcomes, there still exist areas for continued development. The introduction of targeted agents has provided clinicians with additional treatment options in mCRC, however, results have been mixed at best. These novel therapies were designed to interfere with specific molecules involved in the cellular carcinogenesis pathway and ultimately deliver a more focused treatment. Currently, their use in mCRC has been limited primarily as an adjunct to conventional chemotherapy regimens. This review explores the relevant cell-signaling networks in colorectal cancer, provides focus on the current targeted agent armamentarium approved for use in mCRC and explores the usefulness of predictive mCRC biomarkers.

Keywords: biomarkers, colorectal cancer, signaling, targeted therapy

Introduction

Colorectal cancer (CRC) represents a significant health issue as it is the most common gastrointestinal (GI) tract cancer worldwide with over 1.2 million new diagnoses each year [Ferlay *et al.* 2010]. It is the third most common cancer diagnosis in both men and women [Siegel *et al.* 2012]. Each year, there are over 520,000 people newly diagnosed with colorectal cancer in the western world [Ferlay *et al.* 2010]. Between 35–50% of those diagnosed will die from colorectal cancer, making it the second leading cause of cancer deaths affecting both sexes [US Cancer Statistics Working Group, 2009; Ferlay *et al.* 2010; Siegel *et al.* 2012]. Curative approaches are limited in a large proportion of patients as nearly 25% will present with metastatic disease and 40–50% of those diagnosed initially with early-stage disease will eventually develop metastatic disease [Ferlay *et al.* 2007; Siegel *et al.* 2012].

The American Joint Committee on Cancer (AJCC) reports the overall 5-year survival for colorectal cancer at 65.2% [O'Connell *et al.* 2004]. Early-stage disease has a more favorable prognosis and patients are frequently cured with surgical resection alone. Unfortunately most patients with advanced or metastatic disease are not suitable for resection and treatment is part of a palliative, rather than curative, approach. In such a setting, the treatment objectives are to delay disease progression, prolong survival and maintain quality of life.

Despite decades of research and some promising discoveries, the mainstay of metastatic colorectal cancer (mCRC) treatment remains based on cytotoxic chemotherapy agents such as irinotecan or oxaliplatin combined with a fluoropyrimidine and leucovorin (FOLFIRI or FOLFOX regimens) that have both shown modest outcomes when used as first-line therapy [Goldberg *et al.* 2004; Meyerhardt and Mayer, 2005; Tournigand *et al.* 2004]. When 5-fluorouracil (5-FU) and leucovorin were the only therapeutic options, the survival for patients with mCRC was between 10 to 12 months [Erlichman *et al.* 1992; Piedbois, 1998]. The addition of irinotecan or oxaliplatin increased overall survival (OS) to 18 months [Goldberg *et al.* 2004]. The addition of targeted therapies over the past 10 years has improved OS in mCRC to between 20 to 24 months [Fuchs *et al.* 2008; Saltz *et al.* 2008; Tabernero *et al.* 2007; Van-Cutsem *et al.* 2008]. Due the heterogeneous nature of cancer, a number of patients receive targeted treatments with little or no benefit to them [Simon, 2008]. Further analysis of patient nonresponders has led to the discovery of

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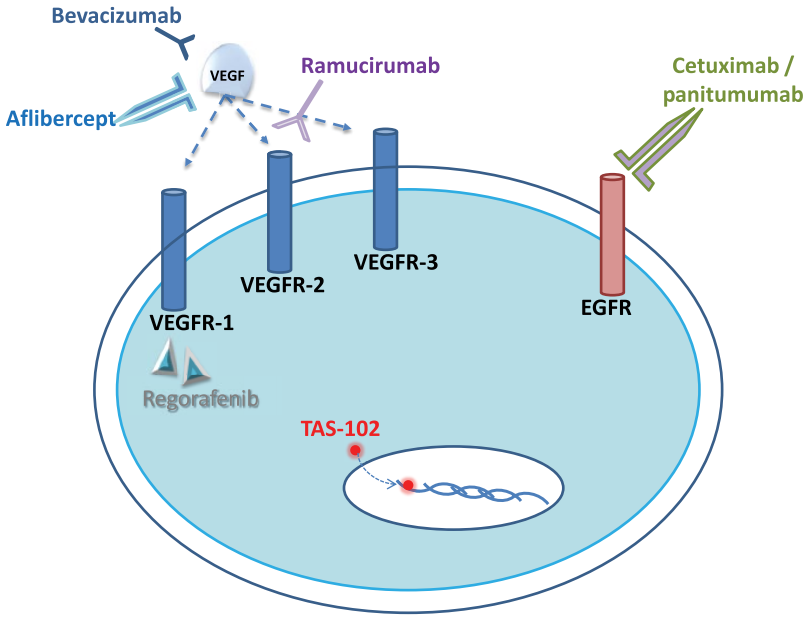

Figure 1. Schematic of an endothelial cell depicting VEGFR-1, VEGFR-2, and VEGFR-3 and the mechanisms of action of the antiangiogenic agents bevacizumab, aflibercept, ramucirumab and regorafenib. Bevacizumab and aflibercept bind to VEGF and interrupt the interaction with VEGF receptors. Regorafenib is a small-molecule multi-kinase inhibitor of which targets include VEGFR-1 and VEGFR-3. TAS-102 consists of trifluridine that is incorporated into DNA, inducing DNA dysfunction, including DNA strand breakage. Cetuximab and panitumumab are anti-EGFR treatments that result in disruption of the MAP kinase pathway.
EGFR, endothelial growth-factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth-factor receptor; DNA, deoxyribonucleic acid; MAP, a protein kinase signaling pathway.

some common genetic alterations in the cancer genome that highlights the need for a more personalized approach [Stuart and Sellers, 2009]. Increased toxicity and treatment costs associated with targeted therapies have further necessitated the identification of diagnostic tools to select for patients who will benefit from such treatments. Currently, our available biomarkers are limited to identifying the patients for whom treatment is not suited, instead of those who would benefit from treatment [Schrag, 2004; Strimpakos *et al.* 2009; Workman *et al.* 2006].

Multiple critical protein-encoding genes and pathways are believed to be responsible for tumorigenesis [Cancer and Atlas, 2012]. Colorectal tumors contain a median 76 mutations, with, on average, 15 of these affecting candidate cancer genes [Vecchione *et al.* 2011; Wood *et al.* 2007]. Increased understanding of the genetic and genomic changes in CRC has helped direct therapies and predict response, as evident in patients with KRAS and BRAF mutations [Sclafani *et al.* 2013; Vaughn *et al.* 2011]. Genetic and epigenetic errors in signal-transduction pathways lead to malignant transformations and have thus emerged as key candidates for molecular-targeted therapies [Tan *et al.* 2009].

There are seven Food and Drug Administration (FDA)-approved targeted therapies in mCRC (Figure 1): the large-molecule monoclonal antibodies (mAbs) (bevacizumab, cetuximab, panitumumab and ramucirumab), a recombinant fusion protein (ziv-aflibercept), a small molecule inhibitor (regorafenib) and a nucleoside analog (trifluridine/tipiracil) [Grothey *et al.* 2013; Hurwitz *et al.* 2004; Van-Cutsem *et al.* 2010a, 2010b, 2012]. This article reviews the recent advances and evidence related to the employment of the FDA-approved targeted therapies in mCRC and explores the available biomarkers [NCI, 2015].

Targeting receptors in colorectal cancer

Vascular endothelial growth factor

Angiogenesis is essential for the normal physiological functions of tissues, however, it also represents a critical process for tumor growth, survival and metastasis [Risau, 1997]. Tumor cells require an extensive supply of new blood

vessels to sustain their rapid growth and spread [Tanigawa *et al.* 1997]. Tumor vascularization occurs through the formation of new vessels from the preexisting vasculature or by insertion of interstitial tissue columns into the lumen of preexisting vessels [Hubbard and Grothey, 2010]. Numerous signaling molecules have been identified in promoting angiogenesis, including vascular endothelial growth factor (VEGF), ephrin, angiopoietin, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) [Folkman and Klagsbrun, 1987; Takahashi *et al.* 1996; Yancopoulos *et al.* 2000]. Among these molecules, VEGF is the most important regulator of the angiogenic process identified to date and has shown markedly increased expression in advanced colorectal tumors [Ferrara *et al.* 2003; Shibuya, 2011; Takahashi *et al.* 1995]. Rapidly dividing tumor cells outgrow their blood supply, creating a hypoxic and nutrient-deficient microenvironment, leading to activation of the hypoxia-inducible factor (HIF) system [Pugh and Ratcliffe, 2003; Tonini *et al.* 2003]. HIF is a critical regulatory factor in the upregulation of VEGF and numerous other proangiogenic mediators (FGF, PIGF and PDGF) from the preexisting vasculature [Eichholz *et al.* 2010; Hoeben *et al.* 2004; Wek and Staschke, 2010]. There are multiple ligands and receptors in the VEGF/VEGF-receptor (VEGFR) axis required for specific binding and the resultant activation of multiple signaling networks [Shibuya, 2001]. VEGF binding initiates a cascade of signaling processes that promote endothelial cell proliferation and migration, remodeling of the extracellular matrix, and increased vascular permeability and dilatation [Ferrara *et al.* 2003]. In addition to this, VEGF has been linked to endothelial progenitor cells involved in neovasculogenesis [George *et al.* 2011]. VEGF is therefore an attractive target when designing and developing drugs to restrict tumor angiogenesis. Numerous anti-VEGF/VEGFR-targeted therapies have demonstrated their potential to inhibit angiogenesis and tumor growth in the preclinical setting [Hicklin and Ellis, 2005].

VEGF (also known as VEGF-A) and its glycoprotein homologues (VEGF-B, VEGF-C, VEGF-D and PIGF) form a subfamily within the PDGF family of growth factors [Meyer *et al.* 1999; Neufeld *et al.* 1999; Shibuya, 2011]. VEGF and its family members mediate their angiogenic effects through differential binding to the three VEGF receptors (VEGFR-1, VEGFR-2, and VEGFR-3) [Matthews *et al.* 1991; Shibuya *et al.* 1990]. VEGF has been identified as the most important regulator of blood vessel formation [Ferrara, 1997; Hicklin and Ellis, 2005]. It is a multifunctional cytokine commonly expressed by tumor cells [Dvorak, 2002]. VEGF binds to both VEGFR-1 and VEGFR-2, inducing endothelial-cell migration and proliferation, in addition to increasing microvascular dilatation, permeability and neovascularization in cancer and other disease processes [Dvorak, 2002; Ferrara *et al.* 2003]. VEGFR-1 and VEGFR-2 are cell-surface-receptor tyrosine kinases (RTKs) expressed predominantly by vascular endothelial cells that activate downstream intracellular kinase-mediated signaling sequences after ligand binding [Hicklin and Ellis, 2005]. Both of these receptors act as signaling molecules during vascular development and have important roles in physiological and pathological angiogenesis in contrast to VEGFR-3, which mainly functions as a regulator of lymphangiogenesis through which it has been linked to promoting metastases [Alitalo and Carmeliet, 2002; Mustonen and Alitalo, 1995; Nathanson, 2003; Roberts *et al.* 2006].

The important role of VEGF-A and its receptor VEGFR-2 in tumor angiogenesis has led to a large amount of research and drug development in mCRC and other malignancies. Therapeutic agents such as bevacizumab and regorafenib have been developed with activity against the VEGF system, either by targeting its ligands, cell-surface receptors or receptor kinases.

Epidermal growth-factor receptor

The epidermal growth-factor receptor (EGFR) has emerged as a captivating therapeutic target due to its key roles in both the regulation of important normal cellular processes and in cancer pathophysiology. EGFR was one of the first growth-factor receptors to be identified and extensively studied [Cohen, 1975]. It is a ubiquitous transmembrane glycoprotein belonging to the ErbB/HER family of receptors, of which it is one of four structurally related receptor tyrosine kinases (RTKs) [Robinson *et al.* 2000]. These include EGFR (or ErbB-1/HER-1), ErbB-2 (HER-2), ErbB-3 (HER-3) and ErbB-4 (HER-4) [Casalini *et al.* 2004].

Ligand binding to the EGFR's extracellular domain triggers receptor homo- or heterodimerization and subsequent autophosphorylation

within its cytoplasmic domain [Scagliotti *et al.* 2004]. Phosphorylation occurs on specific tyrosine residues and creates binding sites for proteins that serve as adaptors of downstream proteins involved in signal transduction [Cohen *et al.* 1981]. Activated signal pathways include RAS/RAF/MAPK, PI3K/AKT, phospholipase C and JAK2/STAT3 [Fiske *et al.* 2009; Hynes and Lane, 2005; Yarden and Sliwkowski, 2001]. Stimulation of these pathways promotes processes responsible for tumor cell growth, proliferation, migration, survival and invasion [Citri and Yarden, 2006; Fischer *et al.* 2003]. There are over 10 ligands identified that bind to EGFR, ErbB-3 and ErbB-4. These include epidermal growth factor (EGF), transforming growth-factor alpha (TGF-α), heparin-binding EGF, amphiregulin, betacellulin, epiregulin, and neuregulin [Hynes and Lane, 2005; Salomon *et al.* 1995; Yarden and Sliwkowski, 2001]. Of these ligands, EGF and TGF-α are thought to be the most important as they selectively bind to EGFR [Jones *et al.* 1999].

EGFR expression is associated with solid tumor growth and is a common component of various malignancies including colorectal, lung, breast, and head and neck [Bonner *et al.* 2010; Nicholson *et al.* 2001; Pirker *et al.* 2009; Spaulding and Spaulding, 2002]. Inappropriate activation of EGFR can occur from receptor or ligand overexpression, gene mutation or amplification and loss of regulatory mechanisms [Kuan *et al.* 2001; Moscatello *et al.* 1996; Pedersen *et al.* 2005]. Abnormal EGFR activity initiates and promotes processes responsible for tumor growth and progression, including cell proliferation and maturation, angiogenesis, invasion, metastasis, and inhibition of apoptosis [Nicholson *et al.* 2001; Rocha-Lima *et al.* 2007; Yarden and Sliwkowski, 2001].

Receptor tyrosine kinase

RTKs are primary mediators of the signal transduction pathways mediating critical cellular processes, such as survival, differentiation and proliferation [Blume-Jensen and Hunter, 2001; ElShamy, 2005]. There are 58 identified RTKs with approximately 20 different classes including the VEGFR, EGFR, Her2/neu (c-erbB2), and c-Kit (stem-cell-factor receptor) [Lemmon and Schlessinger, 2010; Robinson *et al.* 2000]. RTKs are cell-surface allosteric enzymes consisting of a single transmembrane domain that separates an intracellular kinase domain from an extracellular ligand-binding domain [Cadena and Gill, 1992]. Tyrosine kinase activation occurs following ligand binding to the extracellular domain that drives receptor homo- or heterodimerization and autophosphorylation of the receptor complex [Casalini *et al.* 2004]. The phosphorylated receptor complex acts as a site for signaling proteins to assemble, leading to activation of signaling pathways such as RAS/RAF/MAPK, PI3/AKT, STAT3, and protein kinase C [Bogdan and Klämbt, 2001; Schlessinger, 2000]. Intracellular mediators in these pathways transduce signals into the nucleus, affecting DNA synthesis and cell division as well as a variety of cellular processes [Blume-Jensen and Hunter, 2001]. Growth factors or somatic mutations can effect inappropriate RTK activation, consequently promoting tumor-cell proliferation and growth [Arora and Scholar, 2005]. Tyrosine kinases have been the target of biological agents such as mAbs that can interfere with RTK activation or by small-molecule inhibitors that target the intracellular adenosine triphosphate (ATP)-binding site domain.

Targeted therapies

Over the past 10 years, the number of targeted agents used in various malignancies has increased dramatically. Currently there are seven FDA approved targeted agents in mCRC with many more in development and in clinical trials [Chu, 2012]. These targeted agents fall under the broad classification of mAbs, fusion proteins and small molecule inhibitors.

Monoclonal antibodies

MAbs were the first class of targeted agents proven to provide further benefit to patients with mCRC. Currently there are three FDA-approved monoclonal-antibody agents and they act by either binding to the ligand (e.g. bevacizumab) or the extracellular domain of a receptor (e.g. cetuximab and panitumumab) which inhibits tyrosine kinase signal-transduction pathways necessary for cancer development [Cohen *et al.* 2005].

Angiogenesis inhibition through molecular-targeted therapy has been researched for decades with the rationale that disruption of the VEGF–VEGFR axis might prove beneficial in cancer therapy [Folkman *et al.* 1971]. Antibody blockade of VEGF-A was first demonstrated in the early 1990s to suppress human-tumor growth in nude mice [Kim *et al.* 1993]. The antibody treatment selectively suppressed VEGF-A originating

from the tumor and impressively showed significant inhibition of tumor growth without chemotherapy [Kim *et al.* 1993]. Clinical trials with anti-VEGF agents have not been as successful as demonstrated in the murine model, however, they have proven beneficial when in combination with standard chemotherapy regimens.

Bevacizumab. Bevacizumab (Avastin, Genentech/Roche, CA, US) is a recombinant, humanized monoclonal antibody that binds directly to all major isoforms of VEGF-A, forming a protein complex that prevents further binding to VEGF receptors [Ferrara *et al.* 2004]. This neutralizes VEGF signal transduction through both VEGFR-1 and VEGFR-2 and inhibits endothelial cell proliferation and angiogenesis [Ellis, 2006]. Combining an anti-VEGF agent with standard cytotoxic chemotherapy regimens enhances the suppressive effect on tumor-cell growth and the induction of apoptosis in an additive manner [Ellis, 2006]. It also stabilizes tumor vasculature and decreases its hydrostatic pressure, which improves systemic delivery of the chemotherapy agents [Ellis, 2006].

In 2004, the FDA approved bevacizumab as a first-line agent for patients with mCRC based on the results of a randomized, double-blind clinical trial of 813 patients. Bevacizumab, when administered intravenously in conjunction with the IFL regimen (irinotecan, 5-FU bolus, and leucovorin), had a significantly longer median OS than the IFL plus placebo (20.3 *versus* 15.6 months; Table 1). Bevacizumab plus IFL was associated with increased median progression-free survival (PFS) (10.6 *versus* 6.2 months), increased response rate (RR) (44.8% *versus* 34.8%), and longer duration of response (10.4 *versus* 7.1 months) [Hurwitz *et al.* 2004]. In 2006, results from the Eastern Cooperative Oncology Group Study (E3200) led to its approval as a second-line treatment in patients with previously treated mCRC. Following the failure of a prior irinotecan-containing regimen, patients who then received bevacizumab and FOLFOX had increased OS (from 10.8 to 12.9 months; Table 1) and PFS (from 4.7 to 7.3 months; Table 1) [Giantonio *et al.* 2007]. Subsequent studies have validated the addition of bevacizumab to FOLFOX or FOLFIRI regimens in untreated mCRC patients due to their improved RR and PFS [Fuchs *et al.* 2008; Saltz *et al.* 2008]. The most recent FDA approval for bevacizumab was in 2013 for use in combination with a fluoropyrimidine and either irinotecan- or oxaliplatin-based chemotherapy in mCRC patients whose disease had progressed while on a first-line bevacizumab-containing regimen. This decision was based on a large randomized international clinical trial (ML18147), which had 820 patients randomly assigned chemotherapy alone or chemotherapy in combination with bevacizumab. The bevacizumab plus chemotherapy group had a significant improvement in OS compared with chemotherapy alone (11.2 *versus* 9.8 months; Table 1) [Bennouna *et al.* 2013]. There was also a significant improvement in median PFS which increased from 4.0 to 5.7 months with bevacizumab (Table 1) [Bennouna *et al.* 2013].

Treatment with bevacizumab is relatively safe but there are some risks. Early clinical trials suggested that treatment with bevacizumab alone or with chemotherapy resulted in an increased incidence of thrombosis, bleeding, proteinuria, and hypertension [Gordon *et al.* 2001; Kabbinavar and Hurwitz, 2003; Yang *et al.* 2003]. Hurwitz and colleagues found similar adverse effects in mCRC patients receiving bevacizumab therapy but also noted there was a large incidence of patients developing grade 3 hypertension (requiring treatment) [Hurwitz *et al.* 2004]. A recent meta-analysis on the safety of bevacizumab therapy in patients with advanced cancer concluded that there was a slightly higher risk for any severe (grade 3 or 4) adverse event compared with chemotherapy alone [Geiger-Gritsch *et al.* 2010].

Cetuximab and panitumumab. Cetuximab (Erbitux, ImClone, NJ, US) and panitumumab (Vectibix, Amgen, CA, US) are mAbs with FDA approval for use in mCRC. They differ from bevacizumab in their mechanism of action by targeting EGFR, which is associated with tumor progression and a worse prognosis in mCRC and other GI tract malignancies [Kaklamanis and Gatter, 1992; Yasui *et al.* 1988]. Cetuximab is a chimeric human-murine immunoglobulin (IgG1), whereas panitumumab (IgG2) is fully humanized and therefore believed to have less cellular cytotoxicity [Kimura *et al.* 2007; Saltz *et al.* 2006]. Cetuximab and panitumumab bind specifically to EGFR on both normal and tumor cells, and competitively inhibit the binding of EGF, TGF-α and other ligands [Baselga, 2001]. Both mAbs block downstream signaling by binding to the EGFR's extracellular domain, which prevents further ligand binding, sterically hinders dimerization with other RTKs and induces EGFR degradation [Cohen *et al.* 2005; Li *et al.* 2005; Saltz *et al.* 2006].

Table 1. FDA-approved therapeutic monoclonal antibodies used in metastatic colorectal cancer.

Drug	Class	Target	Study (year)	1st or 2nd line	Regimen	Marker	Improvement (months)
Bevacizumab	mAb	VEGF-A	(2004) Hurwitz *et al.* [2004]	1st	IFL	None	OS (15.6–20.3)
Bevacizumab	mAb	VEGF-A	E3200 (2006) Giantonio *et al.* [2007]	2nd (failure of irinotecan regimen)	FOLFOX	None	OS (10.8–12.9) PFS (4.7–7.3)
Bevacizumab	mAb	VEGF-A	ML18147 (2013) Bennouna *et al.* [2013]	2nd (progressed with bevacizumab regimen)	FOLFOX or FOLFIRI	KRAS WT	OS (9.8–11.2) PFS (4.0–5.7)
Cetuximab	mAb	EGFR	BOND (2004) Cunningham *et al.* [2004]	2nd (failure of irinotecan regimen)	FOLFIRI	None	TSR (22.9%) TGD (4.1)
Cetuximab	mAb	EGFR	BOND (2004) Cunningham *et al.* [2004]	2nd (intolerant of irinotecan)	Mono tx	None	TSR (10.8%) TGD (1.5)
Cetuximab	mAb	EGFR	CRYSTAL (2012) Van-Cutsem *et al.* [2007]	1st line (KRAS WT)	FOLFIRI	KRAS WT	PFS (8.4–9.9)
Panitumumab	mAb	EGFR	(2006) Giusti *et al.* [2007]	2nd (failure of FOLFOX/ FOLFIRI)	BSC	None	PFS (7.3–8.0 weeks) OS (0–10%)
Panitumumab	mAb	EGFR	PRIME (2010) Douillard *et al.* [2010]		FOLFOX4	KRAS WT	PFS (8.0–9.6)
Ramucirumab	mAb	VEGF-R2	RAISE Tabernero *et al.* [2015]	2nd (progressed with bevacizumab, oxaliplatin and a fluoropyrimidine)	FOLFIRI	None	OS (11.7–13.3) PFS (4.5–5.7)

EGFR, endothelial growth-factor receptor; VEGFR, vascular endothelial growth factor receptor; FOLFIR, chemotherapy regimen that includes FOL – Folinic acid (leucovorin, calcium folinate or FA), F – Fluorouracil (5FU), IRI – Irinotecan hydrochloride; FOLFOX4, chemotherapy regimen that includes FOL – Folinic acid (leucovorin, calcium folinate or FA), F – Fluorouracil (5FU), OX (Oxaliplatin); IFL, chemotherapy regimen that includes I (Irinotecan), F (Fluorouracil (5FU)), L (Leucovorin); mAb, monoclonal antibody; KRAS Kirsten ras proto-oncogene; WT wild type.

Blocking EGFR activation and subsequent impairment of downstream signaling (RAS-RAF-MAP kinase pathway) results in inhibition of cell growth, induction of apoptosis, decreased matrix metalloproteinase (MMPs) and VEGF production [Vincenzi *et al.* 2010].

There are numerous oncogenic mutations present in CRC which have contributed to the lack of clinical success with targeted therapies in some patient cohorts. Intrinsic or acquired resistances from mutations can lead to a significant variability in clinical response. Identification of the KRAS gene mutation as a marker of impending failure of EGFR-targeted therapy was the first large step in tailoring treatment of individuals [Amado *et al.* 2008; Khambata-Ford *et al.* 2007; Lievre *et al.* 2008; Normanno *et al.* 2009]. The RAS family comprises some small GTPases (hydrolase enzymes that bind and hydrolyze guanosine triphosphate) that are integral constituents of signaling networks contributing to a multitude of vital cellular processes [Bos, 1989]. Frequent oncogenic mutations are found in members of the RAS subfamily (KRAS, NRAS, and HRAS), which lead to tumor development [Fernández-Medarde and Santos, 2011]. KRAS is a critical mediator of EGFR-induced signaling. Activation of EGFR recruits proteins to the cell membrane and causes KRAS to become activated, which results in signaling through the PI3-K/AKT and MAPK (also known as ERK) pathways [Schubbert *et al.* 2007]. KRAS mutants are unable to hydrolyze RAS-GTP to RAS-GDP and thus cannot be restrained, leading to EGFR-independent activation [Schubbert *et al.* 2007].

KRAS mutations have been detected in 40–45% of CRC samples with a high grade of concordance between primary and metastatic sites [Loupakis *et al.* 2009; Vaughn *et al.* 2011]. NRAS and HRAS mutations are less commonly found in CRC (1–3% of samples) [Irahara *et al.* 2010; Vaughn *et al.* 2011]. Most KRAS mutations are missense and affect codons 12 and 13 of exon 2 [Amado *et al.* 2008; Hayashi *et al.* 1995]. The mutation at codon 12 is the most prevalent (80% *versus* 20%) and oncogenic of the two [Guerrero *et al.* 2000]. More recently, KRAS mutations on codons 61 and 146, and exons 3 and 4 have also been reported to

decrease anti-EFGR therapy [Douillard *et al.* 2013; Heinemann *et al.* 2014; Loupakis *et al.* 2009]. In addition to KRAS, there is strong evidence to support BRAF and NRAS mutations inhibiting the effect of anti-EGFR therapy [De Roock *et al.* 2010]. The BRAF mutation has been shown to be a strong negative prognostic factor in CRC [Eklof *et al.* 2013]. The BRAF gene encodes a serine threonine protein kinase which is directly activated by KRAS and leads to stimulation of the MAPK pathway [Di Fiore *et al.* 2010; Wan *et al.* 2004]. The average prevalence of BRAF mutations in colorectal cancer is an estimated 9.6%, with the valine-to-glutamic-acid-amino-acid (V600E) substitution being the most common [Davies *et al.* 2002; Safaee Ardekani *et al.* 2012]. BRAF mutations are considered mutually exclusive with KRAS mutations, as concomitant tumor mutations are extremely rare [Sahin *et al.* 2013]. In a pooled analysis of the CRYSTAL and OPUS randomized clinical trials, BRAF mutations were found to be a marker of poor prognosis but not an effective biomarker predictor in patients treated with anti-EGFR mAbs [Bokemeyer *et al.* 2012]. NRAS is a proto-oncogene from the RAS family and its mutations on exon 2, 3, and 4 have been shown to be effective predictors of anti-EGFR resistance [Douillard *et al.* 2013; Heinemann *et al.* 2014]. PIK3CA mutations on exon 9 and 20 often coexist with KRAS mutations and are associated with poor survival in patients treated with anti-EGFR therapy [Perrone *et al.* 2009; Wu *et al.* 2013].

Anti-EGFR mAbs therefore have minimal if not harmful results in patients with KRAS mutations due to their EGFR-independent activation of oncogenic signaling cascades [Benvenuti *et al.* 2007]. The CRYSTAL study, along with the supportive cetuximab studies, have clearly demonstrated that the presence of KRAS mutations negatively affects the anti-EGFR therapies [Chau and Cunningham, 2009; Dahabreh *et al.* 2011]. This finding led to National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology and the American Society for Clinical Oncology (ASCO) guidelines to recommend restricting anti-EGFR agents to mCRC patients with a wild-type KRAS allele [Allegra *et al.* 2009; Jimeno *et al.* 2009].

The prognostic potential of KRAS mutations in mCRC and its impact on the effectiveness of chemotherapy or anti-VEGF inhibition remains undefined. The KRAS pathway has previously been shown to upregulate angiogenic factors and recently, a study demonstrated KRAS mutant cells to express higher levels of VEGF-A [Downward, 2003; Figueras *et al.* 2013; Zhang *et al.* 2001]. Retrospective analysis of clinical benefit from bevacizumab in patients with wild- or mutant-type KRAS tumors has found comparable benefits in PFS and OS [Hurwitz *et al.* 2009].

Both anti-EGFR treatments appear to be well tolerated, with a low incidence of grade 3 or 4 adverse events. The most common adverse event with cetuximab was an acneiform rash. Other adverse events normally associated with cetuximab therapy include infusion reactions, cardiac events, and hypomagnesemia, as observed in the wild-type KRAS populations of the CRYSTAL, OPUSS and CA225025 trials [Hubbard and Alberts, 2013]. The most common adverse events with panitumumab use were skin rash, hypomagnesemia, paronychia, fatigue, abdominal pain, nausea, and diarrhea [Giusti *et al.* 2007].

In 2004, cetuximab became the first anti-EGFR mAb approved by the FDA for use in mCRC. It was approved as a second-line therapy for use in irinotecan-refractory or intolerant patients with EGFR-expressing tumors. Approval was based on a randomized, two-arm phase II clinical trial (BOND study) of 329 patients. Cetuximab combined with irinotecan significantly improved RRs (22.9% *versus* 10.8%; Table 1) and time to progression (TTP) (4.1 *versus* 1.5 months; Table 1) compared with cetuximab alone [Cunningham *et al.* 2004]. The results demonstrated that interfering with EGFR signaling can resensitize tumors that are refractory to irinotecan. In 2012, the FDA expanded its approval of cetuximab for use as a first-line treatment in patients with KRAS wild type (mutation negative), EGFR-expressing mCRC. The decision was based on retrospective analyses according to KRAS mutation status of tumor samples from patients enrolled in the CRYSTAL trial and two supportive studies (CA225025 and OPUS). The addition of cetuximab to chemotherapy or best supportive care (BSC) resulted in improved OS, PFS and objective response rate (ORR) in patients with KRAS wild-type tumors [Bokemeyer *et al.* 2012]. The use of cetuximab in patients with KRAS mutant tumors provided no benefit, and even potential harm.

The CRYSTAL (cetuximab combined with irinotecan in first-line therapy for mCRC) trial was a phase III open-label, randomized,

multicenter study that included 1217 patients (irrespective of KRAS status) who had not received prior chemotherapy for mCRC. A significant improvement in median PFS was observed for the cetuximab plus FOLFIRI arm compared with the FOLFIRI only arm (8.9 *versus*. 8.1 months) [Van-Cutsem *et al*. 2007]. There were minor but not significant differences in the median OS (19.6 *versus* 18.5 months) and the ORR (46% *versus* 38%) in both trial arms [Van-Cutsem *et al*. 2007]. However, following retrospective analyses of patient subsets for KRAS status, the results were more favorable in the KRAS wild-type patients given cetuximab. An updated survival analysis in 2011 further supported the addition of cetuximab to FOLFIRI as first-line therapy in patients with KRAS wild-type as these patients had increased median PFS (9.9 *versus* 8.4 months; Table 1), median OS (23.5 *versus* 20.0 months) and ORR (57.3% *versus* 39.7%) compared with FOLFIRI alone [Van-Cutsem *et al*. 2011]. The patients with KRAS mutations did not benefit from the addition of cetuximab as they had no improvement in median PFS (8.1 *versus* 7.5 months), OS (15.3 *versus* 15.8 months) and ORR (31.0% *versus* 45.0 %) compared with FOLFIRI alone [Van-Cutsem *et al*. 2011].

CA225025 was an open-label randomized trial that compared cetuximab plus BSC with BSC alone in 572 patients with previously treated EGFR-expressing mCRC. Among patients with wild-type KRAS, cetuximab significantly increased median OS (8.6 *versus* 5.0 months) and PFS (3.8 *versus* 1.9 months). No benefits were observed in the mutant KRAS patients treated with cetuximab.

OPUS (oxaliplatin and cetuximab in first-line treatment of mCRC) was a phase II open-label, randomized study that compared FOLFOX-4 (fluorouracil, leucovorin, and oxaliplatin) plus cetuximab *versus* FOLFOX-4 alone in 337 untreated EGFR-expressing mCRC patients [Bokemeyer *et al*. 2009]. KRAS wild-type patients who received cetuximab plus FOLFOX-4 had increased ORR (57% *versus* 34%) and PFS (8.3 *versus* 7.2 months) compared with those receiving only FOLFOX-4 [Bokemeyer *et al*. 2011]. Median survival time was improved with cetuximab plus FOLFOX-4 but it was not statistically significant (22.8 *versus* 18.5 months) [Bokemeyer *et al*. 2011]. Patients with KRAS mutations who received cetuximab plus FOLFOX-4 had a decreased ORR (34% *versus* 53%) and PFS (5.5 *versus* 8.6 months) compared with those receiving FOLFOX-4 alone [Bokemeyer *et al*. 2011].

A recent comprehensive meta-analysis examined the effect of anti-EGFR mAbs in mCRC patients expressing wild-type KRAS compared with mutant KRAS [Vale *et al*. 2012]. A total of 10 out of 14 RCTs identified had available KRAS status. As expected, there was a positive effect on PFS when anti-EGFR mAbs were used in patients with wild-type KRAS-expressing tumors but not in the mutant KRAS patients. The PFS benefits were confined to trials combining mAbs alongside 5FU-based chemotherapy. There was also no evidence of a PFS benefit when anti-EGFR mAbs were given with bevacizumab.

In 2006, the FDA provided accelerated approval to panitumumab (Vectibix) for the treatment of patients with EGFR-expressing, mCRC with disease progression on or following a FOLFOX/FOLFIRI-containing regimen. The approval was based on the findings of a single, open-label, multinational phase III study that randomized 463 patients to receive panitumumab plus BSC or BSC alone. The median PFS was significantly greater in patients receiving panitumumab compared with BSC alone (8.0 *versus* 7.3 weeks; Table 1) [Giusti *et al*. 2007]. The ORR also favored panitumumab (10.0% *versus* 0%; Table 1). There were 19 partial responses (8%) with a median duration of 17 weeks among the panitumumab group. Retrospective analysis of the study provided further evidence to the importance of KRAS status as clinical benefit was specific to patients with wild-type KRAS tumors given panitumumab monotherapy. The median PFS in the wild-type KRAS group treated with panitumumab was 12.3 weeks compared with 7.3 weeks for BSC [Amado *et al*. 2008]. Panitumumab RRs were also improved in the wild-type KRAS group (17% *versus* 0%). There was no difference in OS between the two study arms, likely due to the crossover design.

The PRIME (panitumumab randomized trial in combination with chemotherapy for metastatic colorectal cancer to determine efficacy) study examined the efficacy and safety of panitumumab in combination with FOLFOX-4. This was a multicenter phase III trial that enrolled 1183 patients with no prior chemotherapy for mCRC. In the wild-type KRAS group, panitumumab plus FOLFOX-4 significantly improved PFS compared

with FOLFOX-4 (9.6 *versus* 8.0 months; Table 1) and nonsignificantly improved the median OS (23.9 *versus* 19.7 months) [Douillard *et al.* 2010]. In the mutant KRAS group, panitumumab plus FOLFOX-4 had a negative effect on both PFS and median OS compared with FOLFOX-4 (15.5 *versus* 19.3 months).

A meta-analysis in 2011 of four randomized clinical studies found significant clinical benefit for panitumumab-based therapy in wild-type KRAS mCRC patients following prior chemotherapy exposure [Ibrahim and Abouelkhair, 2011]. There was an associated 42% improvement in PFS when panitumumab was used as a second-line therapy but no benefit in the first-line setting [Ibrahim and Abouelkhair, 2011].

Both cetuximab and panitumumab are indicated for the treatment of EGFR-expressing, mCRC. Panitumumab approval is for patients with disease progression while on, or following a FOLFOX/FOLFIRI-containing regimen, whereas cetuximab is for use with FOLFIRI as a first-line treatment and also in patients who are irinotecan intolerant or refractory. Panitumumab approval was based on its improvement of PFS, while cetuximab approval was based on ORR. Neither anti-EGFR agent demonstrated a statistically significant benefit in OS, representing a change in the accepted endpoints of a treatment, as previous new agents required an improvement in OS to gain FDA approval [Berlin *et al.* 2006; Tabernero *et al.* 2007].

Ramucirumab. Ramucirumab (Cyramza; Eli Lilly and Co., Indianapolis, IN, US) became the latest FDA-approved mAb on 24 April 2015 [Goel and Sun, 2015]. It is now indicated in combination with FOLFIRI for the treatment of patients with mCRC whose disease has progressed on a first-line bevacizumab-, oxaliplatin- and fluoropyrimidine-containing regimen [Tabernero *et al.* 2015]. Ramucirumab is a recombinant human monoclonal IgG1 antibody that binds and blocks further activity of the human VEGF-R2 with its ligands. Approval was based on the RAISE trial which was a randomized, double-blind, multinational trial enrolling patients with mCRC that progressed during or within 6 months of discontinuation of bevacizumab-, oxaliplatin- and fluoropyrimidine-based combination chemotherapy [Tabernero *et al.* 2015]. The clinical trial consisted of 1072 patients who were randomly allocated (1:1) to receive FOLFIRI plus placebo or FOLFIRI plus ramucirumab (n = 536 per arm) as an intravenous infusion every two weeks. The primary efficacy endpoint of the study was OS. A statistically significant OS improvement was observed in patients receiving FOLFIRI plus ramucirumab compared with those receiving FOLFIRI plus placebo (13.3 *versus* 11.7 months; Table 1). PFS was also significantly improved in patients who received ramucirumab in combination with FOLFIRI (5.7 *versus* 4.5 months; Table 1). The infusion was generally well tolerated, however, thyroid dysfunction was noted in 2.6% of patients.

Fusion proteins

Ziv-aflibercept. In 2012, the FDA approved ziv-aflibercept (Zaltrap; Sanofi and Regeneron Pharmaceuticals, Inc., Tarrytown, NY, US) for the treatment of mCRC that has progressed following an oxaliplatin-containing regimen. Ziv-aflibercept (previously known as aflibercept) is a recombinant fusion protein consisting of VEGF-binding sections from the extracellular domains of human VEGFR-1 and VEGFR-2 attached to the Fc portion of human IgG1 immunoglobulin [Wang and Lockhart, 2012]. Ziv-aflibercept binds to and inactivates circulating VEGF, VEGF-B and PlGF ligands, preventing their interaction with VEGF receptors [Holash *et al.* 2002]. FDA approval was based on the VELOUR trial, an international randomized double-blind study in which 1226 patients received FOLFIRI with either ziv-aflibercept or placebo [Van-Cutsem *et al.* 2012]. These patients all had disease progression during or within 6 months of receiving oxaliplatin-based chemotherapy with or without bevacizumab. A significant improvement in OS (13.5 *versus* 12.1 months; Table 2), PFS (6.9 *versus* 4.7 months; Table 2) and RR (20% *versus* 11%; Table 2) was observed in patients receiving the FOLFIRI plus zib-aflibercept regimen compared with the placebo cohort [Van-Cutsem *et al.* 2012]. Further subgroup analysis found the addition of ziv-aflibercept to FOLFIRI had a trend of increased OS and PFS, regardless of prior bevacizumab use [Allegra *et al.* 2012].

Small-molecule inhibitors

mAbs target circulating growth factors or receptors on the cell exterior whereas small-molecule inhibitors block cell signaling pathways from within. These inhibitors primarily compete with ATP for the ATP-binding site in the hinge region of the kinase receptor by mimicking the hydrogen

Table 2. US Food and Drug Administration (FDA)-approved therapeutic targeted inhibitors used in metastatic colorectal cancer.

Drug	Class	Target	Study (year)	1st or 2nd line	Regimen	Marker	Improvement
Aflibercept	Fusion Ab	VEGF ligand	VELOUR (2012) Van-Cutsem *et al.* [2012]	2nd (failure of oxaliplatin)	FOLFIRI	None	OS (12.1–13.5) PFS (4.7–6.9) RR (11–22%)
Regorafenib	Multikinase	VEGF TIE2	CORRECT (2012) Grothey *et al.* [2013]	3rd (failure of standard therapies)	BSC	None	OS (5–6.4) PFS (1.7–2.0) RR (15–44%)
Trifluridine/ tipiracil	Nucleoside analog	DNA	RECOURSE (2015) Mayer *et al.* [2015]	3rd (failure of standard therapies + biological		None	OS (5.3–7.1) PFS (1.7–2.0)

DNA, deoxyribonucleic acid; VEGF, vascular endothelial growth factor; BSC, best supportive care; FOLFIRI, irinotecan or oxaliplatin combined with a fluoropyrimidine and leucovorin; OS, overall survival; PFS, progression-free survival; RR, response rate.

bonds formed by the adenine ring of ATP [Liu and Gray, 2006]. Other compounds allosterically inhibit the catalytic activity by binding outside the active site [Zhang *et al.* 2009]. Small-molecule inhibitors can either target a single receptor only, such as gefitinib (targets EGFR only), or they can target multiple receptors, as in the use of sorafenib (which targets VEGFR, PDGFR, c-kit, Raf, flt-3 and RET) [Ranson *et al.* 2002; Yau *et al.* 2009]. The most successful use of tyrosine kinase inhibitors in clinical practice has been with gastrointestinal stromal tumors (GISTs) and the inhibition of c-Kit. Most solid tumors have multiple genetic alterations in specific proteins affecting a number of signaling networks making it difficult to target with single inhibitors.

Regorafenib. Regorafenib (BAY 73-4506; Bayer Pharma AG, Berlin, Germany) is an oral multikinase small-molecule inhibitor that blocks several protein kinases involved in tumor growth and angiogenesis which include VEGFR-1, VEGFR-2, VEGFR-3, TIE2, RET, KIT, PDGFR and FGFR [Bhargava and Robinson, 2011; Wilhelm *et al.* 2011]. Additionally, it disrupts the downstream tumor-signaling cascades by binding to the serine/threonine-specific protein kinase BRAF in the MAPK pathway responsible for stimulating cell growth [Wilhelm *et al.* 2011]. In 2012, regorafenib became the first FDA-approved small-molecule inhibitor for use in mCRC when combined with FOLFIRI. This was based on the results of a pivotal phase III, multinational trial called CORRECT, which randomized 760 patients to receive BSC plus either regorafenib or placebo. All the patients had already progressed during or within 3 months of their last standard approved therapies. Regorafenib displayed an increased median OS (6.4 *versus* 5 months; Table 2), PFS (2.0 *versus* 1.7 months; Table 2) and RR (44% *versus* 15%; Table 2) [Grothey *et al.* 2013]. The 1.4 month increase in OS equates a 23% reduction in risk of death in a patient population with a very poor prognosis and few options.

Nucleoside analog

TAS-102 is a combination of trifluridine and tipiracil (LONSURF; Taiho Oncology, Inc., Princeton, NJ, US), the most recent targeted agent to gain FDA approval on 22 September 2015. It is indicated in the treatment of patients with mCRC who have previously been treated with fluoropyrimidine-, oxaliplatin-, and irinotecan-based chemotherapy, an anti-VEGF biologic product, and an anti-EGFR mAb, if RAS is wild type [Mayer *et al.* 2015]. The drug is an oral combination therapy consisting of trifluridine (a thymidine-based nucleoside analog), plus tipiracil hydrochloride (a novel thymidine phosphorylase inhibitor) [Lenz *et al.* 2015]. TAS-102 is a dual-targeting formulation, with its major mechanism of action through trifluridine being incorporated into DNA during DNA synthesis, thereby causing DNA dysfunction and damage [Peters, 2015]. The thymidine phosphorylase inhibitor (tipiracil) prevents the degradation of trifluridine.

Approval was based on a multicenter, double-blind, placebo-controlled trial (RECOURSE study) involving 800 patients with previously treated mCRC [Mayer *et al.* 2015]. The two arms of the study had patients receiving trifluridine/tipiracil (n = 534) plus BSC or matching placebo (n = 266) plus BSC. The inclusion criteria included an Eastern Cooperative Oncology Group (ECOG) status of 0 or 1, absence of brain

metastasis, and absence of ascites requiring drainage in the 4 weeks leading to treatment.

A statistically significant improvement in OS was demonstrated in the trifluridine/tipiracil compared with the placebo arm (7.1 *versus* 5.3 months; Table 2). PFS was also improved in patients randomly allocated to receive trifluridine/tipiracil (2.0 *versus* 1.7 months; Table 2).

The most common adverse drug reactions or laboratory abnormalities were neutropenia (38%), anemia (18%), and thrombocytopenia (5%) [Mayer *et al*. 2015].

Combination therapies

Anti-VEGF anti-EGFR. Paul Ehrlich's magic bullet theory has been realized to some extent with selective-binding agents but the effects are not as overwhelming as anticipated [Winau *et al*. 2004]. The vision of targeted cancer therapies have not reached their full potential; in part due to the complexity of multiple and often redundant molecular pathways that promote oncogenic cellular processes [Tortora *et al*. 2008]. Therefore, it is rationalized that multiple-targeted agents may be required to selectively inhibit the numerous tumor pathways [Johnson and Dippold, 1989]. Preclinical studies had suggested that combined blockade of both VEGF and EGFR may be beneficial [Jung *et al*. 2002; Shaheen *et al*. 2001]. Dual targeting of VEGF and EGFR, two functionally linked and closely related targets could interfere with the molecular feedback loops responsible for acquired resistance and potentially increase the antitumor effects of the individual agents [Saltz *et al*. 2007].

This theory was supported with the results of BOND-2 (bevacizumab and irinotecan compared with cetuximab and bevacizumab alone in irinotecan-refractory colorectal cancer), a randomized, phase II feasibility study of 83 irinotecan-refractory mCRC patients. It demonstrated that the triple combination of irinotecan, cetuximab, and bevacizumab achieved better results in irinotecan-refractory mCRC compared with only cetuximab and bevacizumab. The triple-therapy arm had increased time to tumor progression (7.3 *versus* 4.9 months), objective RR (37% *versus* 20%) and OS (14.5 *versus* 11.4 months) [Saltz *et al*. 2007].

Further studies would not support the good anti-VEGF/EGFR results seen in BOND-2. The CAIRO-2 study, a large multi-institutional clinical trial conducted in the Netherlands, had 755 patients with previously untreated mCRC randomly assigned to receive bevacizumab plus CAPOX (capecitabine and oxaliplatin), or the same regimen accompanied by cetuximab [Tol *et al*. 2009]. Surprisingly, the addition of cetuximab worsened median PFS (10.7 *versus* 9.4 months) and subset analysis demonstrated no improved outcome in patients with wild-type KRAS [Tol *et al*. 2009]. There was even a significant detrimental effect in PFS (8.1 *versus* 10.5 months) to patients with mutated KRAS receiving bevacizumab and cetuximab [Tol *et al*. 2009]. The incidence of adverse events was similar in both treatment groups after the exclusion of cetuximab-related adverse cutaneous effects.

A similar negative outcome was reported in the Panitumumab Advanced Colorectal Cancer Evaluation (PACCE) trial in which previously untreated mCRC patients were randomly assigned to receive chemotherapy (FOLFOX or FOLFIRI) and bevacizumab, either alone or accompanied by panitumumab. The addition of panitumumab to the FOLFOX group reduced both the median PFS (10.0 *versus* 11.4 months) and the median OS (19.4 *versus* 24.5 months) [Hecht *et al*. 2009]. A similar pattern was observed in the smaller FOLFIRI cohort, although the differences were not statistically significant. The PACCE trial was prematurely discontinued due to the negative results and increased adverse events (skin toxicity, diarrhea, infections and pulmonary embolism) in the panitumumab group. There is no obvious reason for the negative effect observed by the combination of an anti-VEGF and anti-EGFR mAbs with standard chemotherapy regimens.

The encouraging results observed in anti-VEGF/EGFR preclinical studies were not validated when examined in randomized trials. The failure of combined targeted therapies illustrates the difficulties and level of understanding we have of molecular oncology. It is possible that there is some interaction between the two antibodies and cytotoxic chemotherapy which negatively affected outcomes in PACCE and CAIRO-2 [Blanke, 2009].

Conclusion

Treatment options for mCRC continue to emerge, however, there remains a number of challenges to overcome. The complicated signaling pathways and network cross-talk involved in tumorigenesis must be more effectively targeted.

There is also the dynamic tumor microenvironment, genetic instabilities and host immune responses to be better understood. Further development of therapies aimed at membrane receptors, intracellular signaling molecules and other protein kinase targets is ongoing. All of these potential targets demonstrate the complexity of cancer and showcase the unlikelihood of finding a 'magical bullet' therapy that will work for all patients. Some promising breakthroughs have been made researching the role of HER2 amplification and microsatellite instability in mCRC patients. As we move forward, further progress in identifying new targeted therapies with associated predictive biomarkers is essential.

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