

# Advances in targeted and immunobased therapies for colorectal cancer in the genomic era

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**Abstract:** Targeted therapies require information on specific defective signaling pathways or mutations. Advances in genomic technologies and cell biology have led to identification of new therapeutic targets associated with signal-transduction pathways. Survival times of patients with colorectal cancer (CRC) can be extended with combinations of conventional cytotoxic agents and targeted therapies. Targeting EGFR- and VEGFR-signaling systems has been the major focus for treatment of metastatic CRC. However, there are still limitations in their clinical application, and new and better drug combinations are needed. This review provides information on EGFR and VEGF inhibitors, new therapeutic agents in the pipeline targeting EGFR and VEGFR pathways, and those targeting other signal-transduction pathways, such as MET, IGF1R, MEK, PI3K, Wnt, Notch, Hedgehog, and death-receptor signaling pathways for treatment of metastatic CRC. Additionally, multitargeted approaches in combination therapies targeting negative-feedback loops, compensatory networks, and cross talk between pathways are highlighted. Then, immunobased strategies to enhance antitumor immunity using specific monoclonal antibodies, such as the immune-checkpoint inhibitors anti-CTLA4 and anti-PD1, as well as the challenges that need to be overcome for increased efficacy of targeted therapies, including drug resistance, predictive markers of response, tumor subtypes, and cancer stem cells, are covered. The review concludes with a brief insight into the applications of next-generation sequencing, expression profiling for tumor subtyping, and the exciting progress made in *in silico* predictive analysis in the development of a prescription strategy for cancer therapy.

**Keywords:** targeted therapies, colorectal cancer, signaling pathways, immune cells, personalized medicine

## Introduction

Colorectal cancer (CRC) is one of the most common cancers in the world and the second-most common cause of cancer-related deaths worldwide.<sup>1</sup> Approximately 1.4 million cases and 694,000 deaths were reported for the year 2012.<sup>2</sup> The increased incidence of CRC in developing nations, eg, countries in Asia, has been associated with smoking, obesity, and changes in lifestyle and diet.<sup>3</sup>

Over the past 10 years, survival in metastatic CRC has improved significantly; this is due to major advances in chemotherapy and targeted drugs. A number of targeted biologic therapies have been approved by the US Food and Drug Administration (FDA), and they are summarized in Table 1. A systematic review on anti-EGFR and anti-VEGF targeted therapies in combination with chemotherapy was recently published.<sup>4</sup> The combination of fluorouracil and leucovorin (FL) resulted in median overall survival durations of approximately 12 months in a Phase III trial first reported in 1994.<sup>5,6</sup> Survival was further improved by another 3 months with irinotecan treatment in combination with FL,<sup>7,8</sup> and 16–20 months with oxaliplatin treatment in combination with FL.<sup>9–11</sup>

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**Table 1** Examples of FDA-approved targeted therapies

Name	Target	Year	Application
Imatinib	ABL, Kit	2003	CML, ALL, GIST
Gefitinib	EGFR	2004	NSCLC
Erlotinib	EGFR	2004	NSCLC, pancreatic
Bevacizumab	VEGF	2004	Metastatic CRC
Sorafenib	VEGFR, PDGFR, Raf1, B-Raf	2005	RCC, liver carcinoma
Cetuximab	EGFR	2005	+ FOLFIRI in metastatic CRC
Panitumumab	EGFR	2006	+ FOLFOX in metastatic CRC
Sunitinib	VEGF, PDGF, Kit	2006	GIST, RCC
Lapatinib	HER2	2007	Breast cancer
Temsirolimus	mTOR	2007	RCC
Everolimus	mTOR	2009	RCC, breast, neuroendocrine, astrocytoma
Crizotinib	ALK, c-MET	2011	ALK-positive NSCLC
Vemurafenib	Raf	2011	Melanoma
Ipilimumab	CTLA4	2011	Melanoma
Aflibercept	Extracellular domain of VEGFR1 and VEGFR2 fused to Fc portion of IgG <sub>1</sub>	2012	+ FOLFIRI in metastatic CRC
Regorafenib	VEGFR2 and -3, RET, Kit, PDGFR, and Raf	2012	Metastatic CRC
Vismodegib	SMO	2012	Basal cell carcinoma
Dabrafenib	BRAF	2013	Metastatic melanoma with BRAF <sup>V600E</sup> mutation
Trametinib	MEK1, -2	2013	Metastatic melanoma with a BRAF <sup>V600E</sup> or BRAF <sup>V600K</sup> mutation
Pertuzumab	HER2	2013	Breast cancer
Blinatumomab	CD10, CD3	2014	B-cell precursor ALL
Pembrolizumab	PDI	2014	Metastatic melanoma with a BRAF <sup>V600E</sup> or BRAF <sup>V600K</sup> mutation
Nivolumab	PDI	2014	Metastatic melanoma
MPDL3280A	PDL1	2014	Metastatic bladder cancer
Olaparib	PARP1 and -2	2014	Ovarian cancer
Idelalisib	PI3K p100δ	2014	CLL in combination with rituximab

**Abbreviations:** ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CRC, colorectal cancer; Fc, fragment, crystallizable; FDA, US Food and Drug Administration; FOLFOX, fluoropyrimidine + leucovorin + oxaliplatin; FOLFIRI, fluoropyrimidine + leucovorin + irinotecan; GIST, gastrointestinal stromal tumor; NSCLC, non-small-cell lung carcinoma; RCC, renal cell carcinoma.

The major step forward in clinical management of metastatic CRC was the combination of FL, irinotecan, and oxaliplatin, which increased the survival rate from 12 to 20 months.<sup>12</sup> In addition to this, the use of these drugs to reduce tumor mass before tumor resection enabled a significant proportion of patients who previously were only treated with chemotherapy to undergo resection of their metastases.

The introduction of biologic agents, such as inhibitors of VEGF and EGFR, further increased survival rates to more than 2 years.<sup>13,14</sup>

The current standard of care for unresectable metastatic CRC combines standard cytotoxic chemotherapy with biologic agents. The biologic agents available for metastatic CRC are categorized into three groups, as shown in Table 2.

**Table 2** Three groups of FDA-approved targeted therapies for metastatic CRCs

Target	Examples	Mode of action	Comments	References
EGFR	Cetuximab	Monoclonal antibody to EGFR	First-line therapy: cetuximab + FOLFIRI or FOLFOX, overall survival was 23.5 months	48, 49
	Panitumumab	Monoclonal antibody to EGFR	First-line therapy: panitumumab + FOLFOX, improved median overall survival of 26 months	50
VEGF	Bevacizumab	Monoclonal antibody to VEGFA	First-line therapy in combination with oxaliplatin-based therapy	19
	Aflibercept	Recombinant protein, decoy receptor for VEGFA, -B, and PIGF	Combination with FOLFIRI resulted in longer median overall survival and progression-free survival	22
Multikinase	Regorafenib	Tyrosine-kinase inhibitor of VEGFR1-3, TIE2	CORRECT trial	24

**Abbreviations:** CRCs, colorectal cancers; FDA, US Food and Drug Administration; FOLFOX, fluoropyrimidine + leucovorin + oxaliplatin; FOLFIRI, fluoropyrimidine + leucovorin + irinotecan.

They are: 1) monoclonal antibodies (mAbs; cetuximab and panitumumab) against EGFR on the surface of tumor cells; 2) antiangiogenic inhibitors targeting tumor vascularization and directed mainly against VEGF or its receptors (the mAbs bevacizumab and aflibercept); and 3) regorafenib, an oral small-molecule inhibitor of intracellular kinases involved in various signaling cascades, such as VEGFR2 and -3, as well as RET, Kit, PDGFR, and Raf kinases.

Many excellent reviews have been written on the application of biological targeted therapies for CRC.<sup>15–17</sup> A brief overview is included here to aid readers to understand the rationale behind these applications.

## Current status with antiangiogenic agents and EGFR inhibitors in CRC Targeting the VEGF system

Bevacizumab is a humanized mAb that binds to VEGFA, preventing it from binding to its target receptors. Bevacizumab has been approved by the FDA for use in combination with the fluoropyrimidine analog leucovorin and topoisomerase I inhibitor irinotecan (FOLFIRI) for the treatment of patients with metastatic CRC who have progressed following an oxaliplatin-containing regimen.<sup>18</sup> Major limitations of anti-VEGF therapy include the need for expensive maintenance therapy and the onset of secondary resistance, as highlighted in several randomized trials.<sup>19–21</sup>

Besides bevacizumab, some progress has been made with new antiangiogenic agents, such as aflibercept and regorafenib. Several other antiangiogenic agents are in clinical development, including ramucirumab, famitinib, vatalanib, SU11248, and vascular targeting agents (VTAs).

Aflibercept is a recombinant fusion protein consisting of the extracellular domain of human VEGFR1 and VEGFR2 fused to the Fc (fragment, crystallizable) portion of human IgG<sub>1</sub>. The VELOUR trial showed that regardless of prior bevacizumab exposure, treatment with FOLFIRI and aflibercept resulted in longer median overall survival and progression-free survival compared to FOLFIRI with placebo. These patients had previously progressed within 6 months of receiving oxaliplatin-containing chemotherapy (with or without bevacizumab).<sup>22</sup>

Regorafenib is a small-molecule multikinase inhibitor. It blocks the ATP-binding site of tyrosine kinases involved in various signaling cascades, such as VEGFR2 and -3, RET, Kit, PDGFR, and Raf kinases, thus preventing downstream signaling.<sup>23</sup> In the CORRECT trial, the median overall survival for patients with metastatic CRC refractory to standard chemotherapy in the regorafenib-treated arm was 6.4 months

compared with 5 months in the placebo-treated arm,<sup>24</sup> and no benefit was observed in 50% of patients. Since there are no known predictive biomarkers for regorafenib, it is unclear why this group of patients did not benefit from this novel multikinase-inhibitor treatment.

Promising results were also demonstrated with ramucirumab, a VEGFR2 inhibitor combined with modified FOLFOX (fluoropyrimidine + leucovorin + oxaliplatin)-6 (mFOLFOX6) as a first-line therapy for metastatic CRC.<sup>25</sup> Results from the Phase III RAISE trial reported at the 2015 American Society of Clinical Oncology Gastrointestinal Symposium showed second-line treatment with the VEGFR2 inhibitor ramucirumab combined with standard FOLFIRI extended survival by 1.6 months versus FOLFIRI alone in patients with metastatic CRC.<sup>26</sup>

Famitinib is another small-molecule tyrosine-kinase inhibitor that primarily targets VEGFR2, c-Kit, and PDGFR. Its safety and efficacy in CRC treatment was tested. While it resulted in improved progression-free survival by 1.3 months (13.1% vs 5.5%), drug-related adverse events led to its discontinuation.<sup>27</sup>

Other oral small multitargeted receptor tyrosine-kinase inhibitors include sunitinib, sorafenib, vatalanib (PTK787/ZK222584), axitinib, cediranib, and brivanib.<sup>28–33</sup> Vatalanib (PTK787/ZK222584) inhibits all three VEGF receptors, as well as PDGFR $\beta$ , c-Kit, and CSF1R. Two Phase II trials in metastatic CRC are ongoing. SU11248 is being evaluated in a number of clinical trials in metastatic breast, renal, and CRCs. Objective responses were observed with SU11248-treated patients with metastatic gastrointestinal stroma tumors resistant to imatinib. This supported further evaluation of this compound in Phase III trials.

Another area of investigation is VTAs, which target the already formed vasculature of tumors rather than inhibiting the growth of new blood vessels.<sup>34</sup> Antitumor activities of VTAs in preclinical models and clinical trials have been comprehensively reviewed.<sup>35</sup>

The first class of VTAs consists of flavone-8-acetic acid and its derivatives, with 5,6-dimethylxanthenone-4 acetic acid being the most promising. In preclinical studies, this acid demonstrated antitumor activity in a wide variety of murine tumors, induced extensive tumor necrosis in patient-derived xenografts, and blocked angiogenesis.<sup>36</sup> However, Phase III trials with non-small-cell lung cancer patients produced negative results.<sup>37</sup>

The second class of small-molecule VTAs are tubulin-binding agents consisting of combretastatins and their analogs.<sup>35</sup> Combretastatin A4 disodium phosphate (CA4DP)

has shown potent vascular disruption and antitumor effects in a wide variety of preclinical tumor models.<sup>38</sup> However, a robust proangiogenic response was observed once treatment stopped. Additionally, tumor pain was reported in three Phase I clinical trials of CA4DP involving a total of 96 patients with advanced cancers.<sup>39–41</sup> In one of these clinical trials, a patient with anaplastic thyroid cancer had a complete response.<sup>39</sup> Several other tubulin-binding agents are also in Phase I clinical development, including AVE8062A, ZD6126, and ABT751.<sup>35</sup>

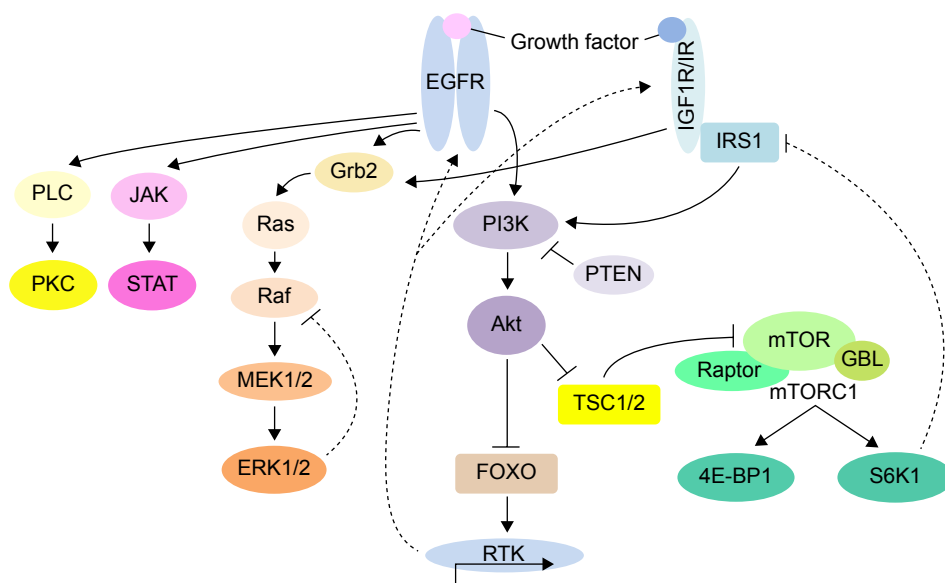
## EGFR-targeted therapies

EGFR, also known as ErbB1 or HER1, is a member of the ErbB transmembrane tyrosine-kinase receptor family. Other members of the family include HER2 and HER3. Ligands that bind EGFR include EGF, amphiregulin, and epiregulin. As shown in Figure 1, ligand binding to the extracellular domain of EGFR leads to activation of the tyrosine-kinase domain in the cytoplasm, activating several intracellular signal-transduction pathways, including Ras/Raf/MEK/ERK, which is mainly involved in cell proliferation, PI3K/Akt, which is involved in cell survival and tumor invasion, JAK/STAT, and phospholipase C.<sup>42</sup> The last two are downstream effectors in a wide range of signal-transduction pathways. Blocking of EGFR with an antibody to EGFR leads to inhibition of these downstream pathways, resulting in reduced

cell proliferation, decreased cell survival, and inhibition of invasion/metastasis.<sup>43,44</sup> However, point mutations in *KRAS* result in constitutive activation of the downstream pathways, such as Raf/MEK/ERK and PI3K/Akt.<sup>45</sup>

Cetuximab (a recombinant chimeric IgG<sub>1</sub> anti-EGFR mAb) treatment provides survival benefit in metastatic CRCs that harbor wild-type *KRAS*,<sup>46,47</sup> and was first approved by the FDA in 2005 for use with FOLFIRI chemotherapy in metastatic CRC. Panitumumab (a fully human IgG<sub>2</sub> antibody) targeting EGFR was approved for use with FOLFOX in 2006 (Table 1).

A retrospective analysis of the Phase II OPUS and Phase III CRYSTAL clinical trials showed that cetuximab combined with a first-line chemotherapy with either FOLFOX4 or FOLFIRI significantly improved response rate, progression-free survival, and overall survival in patients with *KRAS* wild-type tumors.<sup>48</sup> The CRYSTAL study reported overall survival of 23.5 months in patients treated with FOLFIRI and cetuximab compared to 20 months with FOLFIRI alone in previously untreated *KRAS* wild-type metastatic CRC.<sup>49</sup> In the PRIME study, first-line metastatic CRC patients treated with FOLFOX and panitumumab had a 4.2-month improvement in overall survival compared to FOLFOX alone.<sup>50</sup> Cetuximab and panitumumab are currently used in clinical practice in combination with standard combination-chemotherapy regimens or as single agents.



**Figure 1** EGFR and IGF1R signaling pathways.

**Notes:** Binding of extracellular ligands results in autophosphorylation of key tyrosine residues in the C-terminal domain of EGFR, which allows downstream proteins to bind through their Src homology 2 (SH2) domains. This elicits the activation of downstream signaling cascades, including Ras/Raf/MEK/ERK, PI3K/Akt, JAK/STAT, and PLC, which ultimately drive tumor-cell proliferation, survival, and invasion. Growth factor-stimulated IGF1R or IR also induces the activation of the Akt- and ERK-signaling pathways. Akt phosphorylates and inactivates TSC2, leading to activation of the mTOR pathway. Activated Akt induces feedback by inhibiting FOXO transcription factors, thereby downregulating the expression of multiple receptor tyrosine kinases (RTKs) such as EGFR, IGF1R, and IR. mTOR-signaling activation exerts negative feedback by inhibiting IRS1, thereby attenuating PI3K/Akt activation from IGF1R or IR. Negative feedback by ERK also occurs through inhibition of Raf activity, and thus self-limits the activation of ERK signaling.

*EGFR* mutations are rare in CRC, and they are not routinely analyzed in clinical practice. One important finding is that patients with mutation at S492R within the extracellular domain are resistant to cetuximab, but are sensitive to panitumumab.<sup>51</sup> *EGFR* expression is not a useful marker, since its immunohistochemical expression only weakly correlates with treatment response.<sup>52–54</sup> In addition, there is no correlation between *EGFR*-protein expression and *EGFR*-gene amplification.<sup>55</sup> However amplification of *EGFR*<sup>56</sup> or loss of *PTEN* expression<sup>57</sup> may indicate response to cetuximab. It has also been shown that cetuximab provides clinical benefit in patients with high expression of the *EGFR* ligands amphiregulin and epiregulin.<sup>58,59</sup> In contrast, poor prognosis correlated with high expression of *TGF $\alpha$* , another *EGFR* ligand.<sup>60</sup> Expression analysis of *EGFR* and *EGFR* ligands is not routinely implemented in clinical practice.

*KRAS* mutations, which are seen in 35%–40% of CRCs, have emerged as the most important predictive biomarkers in selecting patients who will benefit from cetuximab.<sup>46,47,61,62</sup> Mutations in *KRAS* codons 12 or 13 have been reported in 40% of metastatic CRCs, and are predictive for lack of response to treatment with antibodies to *EGFR*.<sup>63</sup> Mutations in *BRAF*, *NRAS*, and *PI3K* are also associated with poor response to cetuximab.<sup>64</sup> Recent data show patients with mutations in codons 61 and 146 of *KRAS* and codons 12, 13, and 61 of *NRAS* do not benefit from anti-*EGFR* treatment.<sup>63</sup> Therefore, it has been recommended that testing be expanded to include these mutations.<sup>65</sup>

## Resistance mechanisms to cetuximab

As alluded to earlier, one of the major problems in clinical application of anti-*EGFR* inhibitors is acquired drug resistance. A subset of metastatic CRCs responds to the anti-*EGFR* drugs cetuximab and panitumumab, but resistance develops within several months of therapy initiation.<sup>43</sup>

The factors contributing to this acquired resistance are summarized in Table 3.

Mutations in *KRAS* can emerge during treatment with cetuximab in patients with wild-type *KRAS*.<sup>66,67</sup> Emergence of the *EGFR* ectodomain mutant S492R has also been reported.<sup>68</sup> Other studies have demonstrated that oncogenic activation of effectors downstream of *EGFR*, such as mutant *BRAF*,<sup>69,70</sup> *PIK3CA*,<sup>71,72</sup> *PTEN* inactivation,<sup>72,73</sup> and *PTEN* loss,<sup>73</sup> are associated with cetuximab resistance. In addition, approximately 25% of CRC patients with wild-type *KRAS*, *BRAF*, *PIK3CA*, and *PTEN* do not respond to cetuximab, and the resistance mechanism is still unknown.

Other mechanisms that lead to cetuximab resistance include amplification of *MET*,<sup>74</sup> overexpression of *IGF1R*,<sup>75</sup> overexpression of *EGFR* ligands and receptors, such as *ErbB2*<sup>76</sup> and amphiregulin,<sup>77</sup> modulation of *EGFR* by *Src*-family kinases, transactivation of alternative pathways that bypass the *EGFR* pathway, such as *MET* and *IGFR*, ubiquitination, expression of *EGFR* variant III, and induction of *EGFR* translocation.<sup>78,79</sup>

## New-generation anti-*EGFR* therapies

New developments in anti-*EGFR* therapies are summarized in Table 4. Sym004 is a mixture of two mAbs that bind to nonoverlapping epitopes of the *EGFR* extracellular domain. The treatment showed significant clinical activity in *KRAS* wild-type CRC patients who had acquired resistance to anti-*EGFR* therapies.<sup>80</sup> A Phase II trial comparing Sym004 to 5-fluorouracil, capecitabine, or best-support care is currently in progress (NCT02083653) in metastatic CRC patients who are resistant to anti-*EGFR* therapies.

Dual-targeting *EGFR*- and *HER3*-mediated signaling by MEHD7945A, an mAb, was found to be effective in blocking *MAPK*- and *Akt*-signaling pathways and to elicit antibody-dependent cellular cytotoxicity in colon xenograft models.<sup>81</sup>

**Table 3** Possible reasons for acquired resistance to anti-*EGFR* inhibitors and strategies

Factors contributing to acquired resistance to anti- <i>EGFR</i> inhibitors	Strategies to overcome acquired resistance to anti- <i>EGFR</i> inhibitors	References
Acquisition of <i>KRAS</i> mutations	None	66, 67
Emergence of <i>EGFR</i> ectodomain mutation S492R	Mutant is likely to respond to panitumumab relative to cetuximab; use panitumumab instead	68
Increased secretion of <i>TGF<math>\alpha</math></i> and amphiregulin in tumor microenvironment	None	77
Amplification of <i>MET</i> oncogene	Use <i>MET</i> -kinase inhibitors	74
Overexpression of <i>IGF1</i> receptor	Use <i>IGFR</i> inhibitors	75
Amplification of <i>HER2</i>	Dual targeting of <i>EGFR</i> with lapatinib and pertuzumab or combined with neratinib and cetuximab	87
Dimerization of <i>EGFR/HER3</i> and <i>EGFR/HER2</i>	Dual targeting of <i>EGFR</i> and <i>HER3</i>	82, 83

**Table 4** New-generation targeted therapies

Name	Target	Characteristic features	Mode of action	References/ClinicalTrials.gov identifiers
Sym004	EGFR	Composed of two antibodies targeting different epitopes of extracellular domain of EGFR	Anti-EGFR	80; NCT02083653
Imgatuzumab (RG7160 or GA201)		Glycoengineered IgG; at Phase II trial with FOLFIRI	Increased antibody-dependent cellular cytotoxicity of tumor cells	85, 86
MEHD7945A	EGFR and HER3	Dual-action IgG <sub>1</sub> binding to HER3 and EGFR	Blockade of EGFR and HER3, leading to inhibition of MAPK and Akt signaling	83

**Abbreviation:** FOLFIRI, fluoropyrimidine + leucovorin + irinotecan.

It can also overcome acquired resistance to EGFR inhibition by blocking PI3K/Akt- and ERK-signaling pathways.<sup>82</sup> The treatments demonstrated an encouraging safety profile and antitumor activity in a Phase I clinical trial in *KRAS* wild-type CRC patients.<sup>83</sup>

GA201 (RG7160), a humanized anti-EGFR mAb with a glycoengineered Fc region for enhanced binding to FcγRIIIA has been shown to be effective in the killing of *KRAS* wild-type and mutant tumor-cell lines and mouse xenograft tumors.<sup>84</sup> Preclinical studies suggest that GA201 is more effective than cetuximab, and it has shown promising results in two clinical trials with *KRAS*-mutant metastatic CRC patients.<sup>85,86</sup>

HER2 amplification has been found in 2%–3% of metastatic CRC cases, and is associated with up to 36% of cetuximab resistance in *KRAS* wild-type patients. Investigations with patient-derived xenografts have identified HER2 as a therapeutic target in cetuximab-resistant CRCs.<sup>87</sup> An ongoing Phase II trial (NCT01960023) with cetuximab in combination with neratinib, an HER2 tyrosine-kinase inhibitor in metastatic CRC, is in progress.

## New opportunities by targeting other signal-transduction pathways

Examples of clinical trials with a number of targeted agents are summarized in Table 5 and further elaborated in the following sections.

### Targeting *BRAF* mutants

Mutations in genes related to EGFR signaling, including *KRAS*, *BRAF*, and *NRAS*, account for about 60% of cases that develop resistance. B-Raf is downstream of Ras and mutations in *BRAF* have been found in 5%–10% of advanced CRCs and are associated with a very poor prognosis.

Clinical results with vemurafenib (a B-Raf inhibitor) as a single agent in CRC have been disappointing, although the inhibitor is very effective for melanoma patients harboring this mutation.<sup>88</sup> Cell-culture studies have shown that colon

cancer cells treated with an inhibitor of *BRAF*<sup>V600E</sup> mutation were less sensitive to vemurafenib compared to melanoma cell lines with the same mutation.<sup>89,90</sup> Studies in melanoma patients treated with vemurafenib suggest that the inhibition of ERK is necessary to effectively inhibit tumor growth.<sup>91</sup>

Analysis of the phosphorylation status of ERK, EGFR, and Akt in vemurafenib-treated colon cancer cell lines indicated that EGFR and ERK signaling were switched on and that this could be counteracted by cotargeting EGFR.<sup>92</sup> These studies concurred with another study that showed that vemurafenib in CRC caused a rapid feedback activation of EGFR, which supported continued tumor growth.<sup>89</sup> Therefore, the use of B-Raf- and EGFR-inhibitor combinations appears to be a rational strategy. A pilot study using dual B-Raf and EGFR inhibition in *BRAF*-mutated CRC showed a response rate of 13%, but in a subset of patients the response was not durable, lasting less than 1 year.<sup>93</sup>

Another Raf inhibitor, encorafenib, in combination with cetuximab showed a partial response in two of 18 patients.<sup>94</sup> In contrast, improvements were seen with the combination of another B-Raf inhibitor, dabrafenib, with trametinib (MEK inhibitor) and panitumumab, where seven of eight patients achieved stable disease.<sup>95</sup> Further investigations are warranted to clarify the need for ERK inhibition and the role of compensating pathways in these tumors.

### Targeting the MET pathway

MET is the tyrosine-kinase receptor for HGF, and is overexpressed in metastatic colon cancer.<sup>96–98</sup> CRC resistance to anti-EGFR therapies can be driven by *MET* gene amplification in tumor samples. MET amplification was detected in drug-resistant tumor xenografts,<sup>74,99</sup> and was also detected in patient blood samples collected at regular intervals during treatment, thus providing a convenient method for monitoring and predicting resistance. Overexpression of the *MET* gene and MET protein were detected in resistant CRC tumors by fluorescent in situ hybridization and immunohistochemistry, respectively. In addition, antitumor activity was demonstrated

**Table 5** Examples of targeted therapies in clinical development for metastatic CRCs

Target	Examples	Mode of action	Status	References/ClinicalTrials.gov identifiers
VEGFR	Ramucirumab	Anti-VEGFR2	Combined with FOLFIRI, extended survival by 1.6 months	25
	Sunitinib	Multikinase inhibitor	Combined with FOLFIRI	28
	Sorafenib	Multikinase inhibitor	Combined with FOLFOX	29
	Cediranib	VEGFR2 TKI	No significant OS with FOLFOX	32
	Brivanib	Dual VEGFR2 and FGFR1 inhibitor	+ Cetuximab – no clinical benefit	33
	Famitinib	Targets VEGFR2, c-Kit, PDGF	Improved PFS, but discontinued due to adverse events	27
IGF1R	RO5520985	ANG2/VEGFA antibody	Phase II	NCT02141295
	Dalotuzumab	IGF1R monoclonal antibody	+ Cetuximab + irinotecan	NCT00614393
	Cixutumumab	IGF1R monoclonal antibody	+ Selumetinib (MEK inhibitor)	104
	AMG479	IGF1R monoclonal antibody	+ Panitumumab + everolimus	NCT01061788
MET	Tivantinib (ARQ197)	MET-kinase inhibitor	+ Cetuximab	NCT0189257A
	Rilotumumab	HGF monoclonal antibody	+ Panitumumab	NCT00788957
PI3K	BKM120	Small-molecule inhibitor	+ MEK inhibitor	NCT01363232
BRAF	Vemurafenib	BRAF inhibitor	+ Panitumumab	NCT01791309
	LGX818	BRAF inhibitor	+ BYL719 (PI3K inhibitor) + cetuximab	NCT01719380
MEK	Trametinib	Oral MEK1/2 inhibitor	+ Dabrafenib (B-Raf inhibitor) + panitumumab (anti-EGFR)	NCT01750918
	Trametinib	Oral MEK1/2 inhibitor	+ Navitoclax (BCL2-family inhibitor)	NCT02079740
	Selumetinib		+ MK-2206 (Akt inhibitor)	121
	Pimasertib		+ SAR245409 (dual PI3K/mTOR inhibitor)	123
mTOR	MEK162	MEK inhibitor	+ Panitumumab	NCT01927341
WNT pathway	PF-05212384	Dual PI3K/mTOR inhibitor	+ Irinotecan	NCT01347866
	OMP-18R5	Frizzled receptor monoclonal antibody		NCT01345201
WNT pathway	OMP-24F28	Truncated Frizzled 8 receptor fused to the IgG <sub>1</sub> Fc portion		NCT01608867
	PRI-724	Inhibitor of $\beta$ -catenin binding to $\beta$ -catenin-binding complex protein		NCT01606579; NCT01302405
	LGK974	Inhibitor of acyltransferase Porcupine		NCT01351103
	WNT974	Wnt inhibitor	Combined with LGX181 (B-Raf inhibitor) + cetuximab	NCT002278133
Sonic Hedgehog	Vismodegib	Smoothened antagonist	Combined with bevacizumab and FOLFOX or FOLFIRI	NCT00636610
SMO	LEQ506	Smoothened antagonist		NCT01106508
NOTCH	R04929097	$\gamma$ -Secretase inhibitor	Combined with gemcitabine	149; NCT01198535; NCT01270438
	PF-03084014	$\gamma$ -Secretase inhibitor	Combined with irinotecan	NCT00878189; 151
CR3	IMPRIME	$\beta$ -Glucan polymer, which binds to CR3	Combined with cetuximab Cetuximab activates complement, providing iC3b for opsonization of tumor cells; IMPRIME binds to CR3 and leads to cytotoxicity of opsonized tumor cell	NCT01309126

**Abbreviations:** CRCs, colorectal cancers; Fc, fragment, crystallizable; FOLFOX, fluoropyrimidine + leucovorin + oxaliplatin; FOLFIRI, fluoropyrimidine + leucovorin + irinotecan; OS, overall survival; PFS, progression-free survival; TKI, tyrosine-kinase inhibitor.

using a combination of the clinically approved MET inhibitor crizotinib, and anti-EGFR demonstrated efficacy in two patient-derived xenografts, thus providing proof of concept that combination of MET- and EGFR-inhibitor therapies offers novel opportunities to overcome anti-EGFR resistance. Other studies have also demonstrated that activation of the

HGF-dependent MET-signaling pathway contributes to cetuximab resistance in colon cancer.<sup>100</sup> In addition, constitutive MET activation was shown to be due to the formation of an MET/SRC/EGFR complex.<sup>100</sup> This provides a rationale for combinatorial inhibition of EGFR and MET or EGFR and SRC in therapy targeting colon cancer.

A single-arm Phase II study of tivantinib (ARQ 197; MET-kinase inhibitor) combined with cetuximab is in progress in locally advanced or metastatic CRC patients with wild-type *KRAS*, MET-amplified and EGFR inhibitor-resistant tumors (NCT01892527). Another clinical trial (NCT00788957) in progress is testing the combination of an anti-HGF mAb (MG 102; rilotumumab) with panitumumab.

MET has been found to be upregulated in response to VEGF-pathway inhibition, implicating it in antiangiogenic therapy resistance. In a preclinical CRC tumor xenograft model, potent antitumor activity was demonstrated in 80% of tumors with cabozantinib, a dual c-MET and VEGFR2 inhibitor.<sup>101</sup> The antitumor activity was attributed to the inhibition of angiogenesis, inhibition of Akt, and downregulation of genes involved in the PI3K pathway. A clinical trial of cabozantinib in refractory metastatic CRC is currently being activated.

## Insulin-like growth factor-signaling pathway

The IGF-signaling pathway plays a key role in cell growth and metabolism. Overexpression of IGF1 and IGF1R has been found in many cancers, including CRC.<sup>102</sup> Binding of IGF1 to its receptor induces the activation of IRS1 and -2, which then activate several intracellular signaling pathways, including the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways. This leads to cell-cycle progression, cell proliferation, and cell survival. Therefore, blockade of this pathway would reduce cell proliferation and growth.

Indeed, it has been demonstrated that a combination of OSI-906 (IGF1R inhibitor) with selumetinib (MEK1/2 inhibitor) exerted synergistic antiproliferative activity in 13 colon cancer cell lines and an in vivo xenograft model.<sup>103</sup> A Phase I clinical trial targeting these two pathways with selumetinib (MEK1/2 inhibitor) and cixutumumab (IGF1R mAb) in 30 patients with a variety of tumor types, including 13 patients with gastrointestinal tumors (colorectal, pancreatic, and biliary) and four patients with thyroid cancers, is in progress.<sup>104</sup> The combination appears to be well tolerated in these patients.

Activation of IGF1R has been implicated in resistance of lung cancer cells and colon cancer cells to erlotinib, an EGFR tyrosine-kinase inhibitor,<sup>105</sup> further supporting the need to block the IGF1R-signaling pathway when administering anti-EGFR-targeted therapies. A clinical trial with a combination of AMG479 (IGF1R mAb) and panitumumab (anti-EGFR) is in progress (NCT01061788). A Phase II clinical trial with dalotuzumab (MK0646; anti-IGF1R) and

cetuximab in combination with chemotherapy (irinotecan) is also ongoing (NCT00614393).

## Targeting MEK- and PI3K-signaling pathways

The Ras/Raf/MEK/ERK and PI3K/Akt/mTOR signaling pathways are among the best-characterized kinase cascades in cancer cell biology.<sup>105,106</sup> Class I PI3K consists of a regulatory p85 subunit and a catalytic p110  $\alpha$ -subunit. The p110 catalytic subunit occurs in four isoforms, designated as p110 $\alpha$ , - $\beta$ , - $\gamma$  and - $\delta$ . Three isoforms consist of the regulatory p85 subunit and p110 $\alpha/\beta/\delta$  subunit, while the fourth isoform consists of p110 $\gamma$  and a regulatory subunit – p101. A phase I clinical trial with an oral panclass IPI3K inhibitor, pictilisib, has shown that it is safe and tolerable, so further investigation is warranted.<sup>107</sup>

Idelalisib is a first-in-class, oral PI3K $\delta$ -specific inhibitor approved for the treatment of chronic lymphocytic leukemia in combination with rituximab. In addition to idelalisib, two other oral PI3K $\delta$ -specific inhibitors in development are TGR-1202 and AMG-319 (NCT01767766, NCT01300026).

The activation of these pathways is triggered by growth factors or activating mutations of oncogenic kinases, such as K-Ras, N-Ras, B-Raf, and PI3K, resulting in enhanced cell survival, proliferation, and motility. Mutations in *KRAS*, *NRAS*, or *BRAF* (all upstream of MEK complexes) have been found in approximately 40%, 1%–3% and 5%–15% of CRC tumors, respectively.<sup>108</sup> Mutations of the PI3K catalytic p110 $\alpha$  subunit have been found in 10%–20% of CRC tumors. Activating mutations occur in two hotspots located in exon 9 (E542K, E545K) and exon 20 (H1047R) in approximately 15% of CRCs.<sup>109</sup> Only exon 20 mutations (about 25% of total), but not exon 9 mutations, are associated with clinical resistance to anti-EGFR mAbs.<sup>64,110</sup> A third of CRC tumors have co-occurrence of both *KRAS* and *PI3K* mutations.<sup>108</sup> Targeting PI3K alone has not been found to be effective for treating solid cancers.<sup>111,112</sup> This may be due to negative-feedback loops, compensatory networks, and cross talk between pathways.

Activating *PIK3CA* mutants can induce phosphorylation of Akt, which then promotes cell growth and suppresses apoptosis in CRCs. A downstream component of the PI3K-signaling pathway is mTORC1, which plays a major role in nutrient uptake and cell growth.<sup>113</sup> mTOR is the catalytic component of the mTORC2 and mTORC1 multiprotein-kinase complexes. Akt phosphorylates mTORC1, which then drives protein synthesis. The signaling events in both these pathways intersect, and they regulate each other and their downstream



functions. The extent of this cross talk is clinically important, and has provided insights into negative-feedback loops and compensatory pathways. Aberrant hyperactivation of these pathways leads to tumorigenesis. Therefore, these pathways are attractive targets for inhibition, and a number of targeted agents are in clinical development.

Signaling through an mTOR-dependent negative-feedback loop results in the inhibition of PI3K signaling. When mTORC1 is inhibited by rapamycin or its derivatives (rapalogs), this negative-feedback loop is disrupted, leading to activation of PI3K and its effector Akt.<sup>114</sup> PI3K can then act on Ras to promote Ras-dependent ERK activation.<sup>115</sup> The binding of Ras to PI3K activates the EGFR- and FGF2-signalling pathways. As shown in Figure 1, activated mTOR also phosphorylates the downstream substrate S6K1, which then phosphorylates the IRS protein and induces IRS degradation, resulting in less interaction between IRS with IGF1R and insulin receptors. Disruption of this feedback loop by mTORC1 inhibitors will enhance the stability of IRS, which can then interact with IGF1R and insulin receptors. This illustrates the alternative pathways that bypass mTORC1 inhibition. Therefore, dual blockade of PI3K and mTORC1 appears to be a rational step to inhibit tumor survival.

A number of dual PI3K/mTOR inhibitors have been discovered and are currently in clinical development.<sup>116</sup> Some compounds have also been developed to inhibit both class I PI3K isoforms and mTORC1 and mTORC2.<sup>117</sup> Combination therapies with dual PI3K/mTOR inhibitors and chemotherapy are also being investigated, whereby a Phase II trial is testing PF-05212384 (dual PI3K/mTOR inhibitor) with or without irinotecan (NCT01347866), and another trial is comparing PF-05212384 plus irinotecan versus cetuximab plus irinotecan (NCT01925274).

Due to multiple points of cross talk and negative-feedback loops, inhibition of the PI3K/Akt/mTOR pathway gives rise to activation of the Ras/Raf/MEK-signaling pathway and vice versa.<sup>114,118</sup> Numerous preclinical models in Ras-driven tumors have shown that single-agent inhibitors of downstream Ras/Raf/MEK/ERK and PI3K/Akt/mTOR pathways do not lead to significant antitumor activity, due to multiple points of cross talk, negative-feedback loops, and redundancy.<sup>115,119</sup> One example is the finding that selumetinib (MEK inhibitor) treatment upregulated Akt phosphorylation,<sup>120</sup> indicating cross talk between these two pathways and suggesting simultaneous inhibition of both pathways may be of clinical benefit. A Phase I clinical trial with MK-2206 (an allosteric inhibitor of Akt) and selumetinib (a non-ATP competitive inhibitor of MEK) demonstrated that

this combination had antitumor activity at tolerable doses, and thus further investigation is warranted.<sup>121</sup> Another promising potential combination is to cotarget MEK and PI3K pathways. Table 5 shows a summary of ongoing Phase II and III clinical trials with MEK and PI3K inhibitors.<sup>116,122</sup> A phase IB study of the MEK inhibitor pimasertib and the PI3K/mTOR inhibitor SAR245409, however, found this combination to be toxic.<sup>123</sup>

One possible mechanism for the intrinsic anti-EGFR resistance in CRC patients harboring *KRAS* mutations may be the direct activation of the MAPK-signaling pathway.<sup>61,62,124–126</sup> Therefore, one possible strategy is to cotarget the MAPK-signaling pathway. One example is the use of panitumumab and MEK162 in a Phase II clinical trial (NCT01927341).

Resistance to B-Raf inhibitors in *BRAF*-mutated tumors has been found to be due to PI3K activation.<sup>127</sup> However, resistance can be overcome by combining B-Raf inhibitors with chemotherapy<sup>120</sup> or with PI3K inhibitors, as shown in a murine model of *BRAF*-mutation-positive CRC.<sup>128</sup> In cultured *BRAF*-mutated cell lines, resistance to vemurafenib can also be overcome by combining vemurafenib with PI3K inhibitors.<sup>129</sup> The importance of the PI3K pathway in *BRAF*-mutated tumors is now being tested in a clinical trial with a combination of LGX818 (B-Raf inhibitor) and cetuximab with and without the PI3K inhibitor BYL719 (NCT01719380).

## Targeting the Wnt-signaling pathway

Wnt is a family of 19 secreted glycoproteins that accumulate in the extracellular matrix and activate cell-surface receptor-mediated signal-transduction pathways to regulate a range of cellular processes, such as cell migration, proliferation, cell polarity, and development. The Wnt/ $\beta$ -catenin pathway is activated when a Wnt ligand binds to the Fz receptor and its coreceptor LRP6 or its close relative LRP5. The formation of a likely Wnt-Fz-LRP6 complex together with the recruitment of the scaffolding protein Dvl results in LRP6 phosphorylation and the recruitment of the Axin complex to the receptors. In the canonical pathway, when Wnt signaling is not active,  $\beta$ -catenin is recruited into the Axin complex, which consists of APC, Axin and GSK3. This complex is then degraded following phosphorylation of  $\beta$ -catenin. In contrast, in Wnt-activated cells, the receptor occupancy by Wnt ligands in cells inhibits the kinase activity needed for the destruction of the complex, resulting in accumulation of  $\beta$ -catenin in the cytoplasm and its translocation to the nucleus. The accumulating  $\beta$ -catenin in the nucleus combines with the T-cell factor (TCF) transcriptional regulator complex

to activate the transcription of many target genes, such as *MYC*, *CCND1*, *BIRC5*, and *VEGF*, and metalloproteinases that promote cell proliferation, survival, and angiogenesis.<sup>130</sup> Association of deregulated Wnt/ $\beta$ -catenin signaling with cancer and particularly with CRC has been well documented, and thus blockade of this pathway has been of great interest for cancer therapy.<sup>131,132</sup>

The tumor-suppressor gene *APC* has been found to be mutated in approximately 80% of CRCs. Mutational inactivation of *APC* leads to hyperactivation of the Wnt pathway. In the Cancer Genome Atlas Network study, 16 different altered Wnt-pathway genes were found in CRC.<sup>108</sup> The consequence of aberrant Wnt activation is a rapid proliferation of the stem cell compartment in the crypts and the generation of adenomas.

A number of Wnt-signaling pathway inhibitors have been described to date.<sup>133</sup> The first class of Wnt-pathway inhibitors are small molecules that block TCF/ $\beta$ -catenin signaling by disrupting TCF- $\beta$ -catenin interaction<sup>134</sup> or  $\beta$ -catenin-co-activator (CBP) interaction, such as ICG-001.<sup>135</sup> The disruption of TCF- $\beta$ -catenin interaction inhibits production of prostaglandin E<sub>2</sub>, resembling the action of nonsteroidal anti-inflammatory drug, which has been found to be beneficial in CRC prevention and therapy.<sup>136,137</sup>

A second class of Wnt-pathway inhibitors blocks binding of  $\beta$ -catenin to the Axin-binding complex. One such small-molecule inhibitor is PRI-724, which is currently in clinical trials (NCT01606579, NCT01302405), in combination with a modified FOLFOX6 regimen for refractory colon cancer patients.<sup>138</sup>

A third class of Wnt-pathway inhibitors targets the activity of Porcupine, a membrane-bound acyltransferase that is responsible for secretion of Wnt ligands.<sup>139,140</sup> Clinical trials are ongoing with LGK974 (inhibitor of acyltransferase Porcupine; NCT01351103). A study of WNT974 (Porcupine inhibitor that blocks the palmitoylation and secretion of Wnt ligands) in combination with LGX818 (B-Raf inhibitor) and cetuximab (EGFR inhibitor) in patients with *BRAF*-mutant metastatic CRC and Wnt-pathway mutations has also been reported.<sup>141</sup>

A fourth class of inhibitors stabilize the Axin protein, thereby promoting  $\beta$ -catenin degradation even in cancer cells that have lost APC function.<sup>139,140</sup> One such inhibitor is JW55,<sup>142</sup> a small-molecule tankyrase 1/2 inhibitor that can inhibit the growth of colon cancer cells in both cell cultures and animal models.

Finally, several mAbs have been developed to bind and sequester either Wnt ligands or Fz receptors. OMP-18R5, an mAb that binds to Fz receptors 1, 2, 5, 7, and 8, and blocks

$\beta$ -catenin signaling in response to Wnt3a, inhibited growth of human tumor xenografts in several cancers, including colon, breast, pancreatic, and lung cancers.<sup>143</sup> Clinical trials for OMP-18R5 are ongoing (NCT01345201).

OMP-24F28 is a truncated Fr8 receptor fused to the IgG<sub>1</sub> Fc portion, which has the ability to sequester the Wnt ligand. Antitumor activity was demonstrated in preclinical models, and Phase I trials in patients with a variety of advanced solid tumors are in progress, including three Phase III trials in combination with chemotherapy for pancreatic, hepatocellular, and ovarian cancers (NCT01608867).<sup>144</sup>

## Targeting the Notch-signaling pathway

The Notch-signaling pathway consists of four receptors (Notch 1, 2, 3, and 4) and at least five ligands: Jagged 1, Jagged 2, Delta 1, Delta 3, and Delta 4.<sup>145</sup> In the canonical Notch pathway, the binding of a ligand to the Notch receptor results in a cascade of proteolytic cleavages mediated first by a metalloprotease and then by a  $\gamma$ -secretase,<sup>145</sup> resulting in release of a constitutively active intracytoplasmic Notch fragment that is then translocated to the nucleus.<sup>146</sup> The Notch-signaling pathway has been shown to be aberrantly activated in colon adenocarcinoma,<sup>147</sup> and thus blockade of this pathway appears to be a rational therapeutic strategy. However, single-agent treatment of metastatic CRC patients with the  $\gamma$ -secretase inhibitor RO4929097 showed little to no effect.<sup>147</sup>

Treatment of colon cancer cell lines with a  $\gamma$ -secretase inhibitor renders the cell lines more sensitive to chemotherapeutic treatments, such as with oxaliplatin, 5-fluorouracil, or SN-38 (irinotecan).<sup>148</sup> Whether this in vitro study can be extrapolated to in vivo is still unknown. A total of 18 patients with advanced solid tumors are currently enrolled in a Phase I study of the oral  $\gamma$ -secretase inhibitor RO4929097 in combination with gemcitabine.<sup>149</sup> In addition, two ongoing trials are testing the efficacy of RO4929097 in CRC in combination with other chemotherapeutic drugs (NCT01198535 and NCT01270438). Another  $\gamma$ -secretase inhibitor, PF-03084014, has been evaluated in a CRC-explant model, where patient tumors were explanted into nude mice.<sup>150</sup> A PF-03084014-plus-irinotecan combination was found to be more effective than a  $\gamma$ -secretase inhibitor or irinotecan administered as single agents. PF-03084014 (NCT00878189) has undergone one clinical trial and shown promising results, with durable tumor responses, thus warranting further evaluation.<sup>151</sup>

## Targeting the Hedgehog-signaling pathway

The Hedgehog (Hh)-signaling pathway plays a crucial role in embryonic development, and is generally silenced in adults

but reactivated during tissue repair and regeneration.<sup>152–154</sup> Aberrant Hh-pathway activation has been found in a variety of cancers, such as glioma, medulloblastoma, and gastric, pancreatic, breast, and basal cell cancers.<sup>155–160</sup>

Binding of Hh ligands to the receptor Patched leads to derepression of Smoothened (Smo) and modulation of the transcription factors Gli1, Gli2, and Gli3.<sup>161,162</sup> It has been shown that sonic Hh (Shh), which is the best-studied ligand of the Hh-signaling pathway, increases growth of xenograft colon tumors<sup>163,164</sup> and Shh–Gli1 signaling contributes to the enhancement of tumor stemness.<sup>165</sup> Therefore, Hh signaling results in the initiation, progression, and relapse of cancer, including the acquisition of the cancer stem cell phenotype. Gli3 and Shh are expressed in subsets of CRC,<sup>166</sup> and thus are attractive therapeutic targets for development of anticancer agents. Efforts toward developing Hh-pathway inhibitors led to the discovery of the orphan G-protein-coupled receptor GPR39.<sup>167</sup>

Several targeted agents against Hh-signaling pathways have been developed, and most of these agents antagonize Smo. The Smo inhibitors in clinical trials include vismodegib, BMS-833923, IPI-926, LDE-225, PF-04449913, LEQ 506, and TAK-441, and are summarized in Banerjee and Hadden.<sup>168</sup>

Vismodegib was the first Hh-pathway inhibitor approved in the US (in 2012) for the treatment of metastatic or locally advanced basal cell carcinoma. A Phase II randomized clinical trial of vismodegib in combination with FOLFOX1 or FOXFIRI in 195 patients with previously untreated metastatic CRC found no extension of progression-free survival.<sup>169</sup>

Another Smo antagonist, IPI-926, an orally bioavailable derivative of cyclopamine, has demonstrated antitumor activity in a mouse pancreatic cancer model, but a Phase II clinical trial in combination with gemcitabine was stopped early, due to adverse side effects. Different combinations of IPI-926 with chemotherapy in clinical trials were summarized in a recent review.<sup>170</sup>

The Smo antagonist LDE-225 has shown partial response in medulloblastoma and complete response in basal cell carcinoma. GANT61 is a small molecule that inhibits binding of GLI1 and GLI2 to the promoters of target genes, and has been found to kill colon cancer cells.<sup>171</sup>

Initial preclinical studies with the Smo antagonist vismodegib showed that resistance can develop due to *SMO* mutations that promote Hh-pathway activation. An *SMO* mutation at position 1,697, changing codon 473 from Asp to His and conferring resistance to vismodegib, has been described.<sup>172</sup> Another mechanism for resistance is the amplification of the GLI2 transcription factor and the Hh-target gene *CCND1*.<sup>173</sup> Lastly, upregulation of the compensatory

pathway IGF1R/PI3K was observed in LDE-225-resistant tumor samples. In a mouse medulloblastoma model, addition of the PI3K inhibitor BMK120 or the dual PI3K–mTOR inhibitor BEZ235 to an Smo antagonist delayed or prevented the development of drug resistance.<sup>174</sup> Therefore, the combination of an Smo antagonist and dual PI3K–mTOR inhibitor appears to be a rational combination for cancer therapy.

## Targeting the TGFβ-signaling pathway

The role of TGFβ as a tumor promoter or tumor suppressor is still debatable, as it has a differential role in early and late-stage cancer. Targeting the tumor microenvironment by inhibiting the TGFβ pathway appears to be a rational approach, since TGFβ functions to generate a favorable microenvironment for tumor growth and metastasis throughout all the steps of carcinogenesis. Many TGFβ-pathway inhibitors have been discovered, some of which are now in clinical development. These inhibitors include fresolimumab, TβM1, and PF-03446962. An excellent review on TGFβ inhibitors has been published.<sup>175</sup> To date, reliable predictive biomarkers of response to TGFβ inhibitors and appropriate combinations with chemotherapy have not been identified. As with many of the targeted therapies, until predictive markers of response are available to guide the selection of patients, the efficacy of TGFβ inhibitors will be limited.

## Activation of death-receptor 4 and 5 pathways

Death receptors (DRs), including TNFR1, Fas, DR4, and DR5, are attractive targets for cancer therapy. These DRs have an intracellular death domain that transmits a death signal upon binding of their cognate ligands, such as TNFα, FasL, or TRAIL. TRAIL induces apoptosis through engaging DR4 and/or DR5 expressed on the plasma membrane of target cancer cells, and triggers signal transduction via the assembly of a death-inducing signaling complex, activation of a caspase cascade, and cleavage of cellular proteins.<sup>176</sup> Multiple clinical trials have been conducted to evaluate the antitumor activity of recombinant human TRAIL (rhTRAIL), which binds DR4 and DR5 and is used in combination with various chemotherapies or targeted therapies<sup>177,178</sup> Agonistic antibodies that target DR4/5 in combination with other therapies are also being trialed. Examples include the application of dulanermin (Apo2L/rhTRAIL) in combination with cetuximab + irinotecan or bevacizumab + FOLFIRI (NCT00671372) and bevacizumab + FOLFOX (NCT00873756). In vitro and in vivo studies have demonstrated that conatumumab (AMG-655) a fully human IgG<sub>1</sub> mAb that binds to the extracellular domain of DR5, induces

apoptosis in CRC and pancreatic cancer cell lines, as well as in xenograft tumors. Preclinical studies also indicated that conatumumab enhances the antitumor activity of agents, such as irinotecan and gemcitabine.<sup>179</sup> It has also been tested in Phase II clinical trials in combination with FOLFIRI (NCT00813605), with mFOLFOX6 + bevacizumab (NCT00625651), and with panitumumab (NCT00630786) for metastatic CRC patients.

## Immunobased therapies to enhance antitumor immunity

Normally transformed cells are eliminated by the immune system, and cells of both the adaptive (T cells) and the innate (macrophages, natural killer cells) immune system contribute to this. Established tumors, however, subvert the immune system to produce tumor-protective and tumor-promoting

factors. Immunotherapy was named the breakthrough of the year in the last 2013 issue of the journal *Science*. Cancer immunotherapy attempts to rebalance the immune response by inhibiting the immunosuppressive factors in the tumor microenvironment and/or by enhancing the antitumor functions of the immune system.

## Enhancing T-cell function

The first approach is to enhance the T-cell response by using immune-checkpoint inhibitors. Immune-checkpoint inhibitors in clinical trials for CRC patients are summarized in Table 6. CTLA4 is a negative costimulatory molecule expressed on both T cells and regulatory T cells. It acts by inhibiting T cells and promoting regulatory T-cell function. Promising results with combination of mAbs against CTLA4 and PD1 have been demonstrated in advanced melanoma.<sup>180</sup>

**Table 6** Current clinical trials with immune-checkpoint inhibitors in CRCs

Drug	Mechanism of action	Phase of study/ comments	References/ClinicalTrials.gov identifiers
Tremelimumab	CTLA4 antibody		181
MPDL3280A	PDL1 antibody	Combined with bevacizumab and/or chemotherapy	184
Nivolumab	PD1 antibody	One of 13 patients survived >3 years	183
Pembrolizumab (MK-3475)	PD1 antibody	II	NCT01876511
Ipilimumab	CTLA4 antibody	I/II	NCT02239900
Pembrolizumab	PD1 antibody	Ib	NCT02054806
MEDI4736	PDL1 antibody	I/II	NCT01693562
MEDI6469	OX40-agonist antibody	I/II	NCT02205333
Nivolumab	PD1 antibody; trials done ± ipilimumab	I/II	NCT02060188
Urelumab (BMS-663513)	4-1BB/CD137 antibody	I/II	NCT02253992
Urelumab (BMS-663513)	4-1BB/CD137 antibody	I	NCT0211008
Urelumab (BMS-663513)	4-1BB/CD137 antibody	I	NCT01471210
PF-05082566	4-1BB/CD137 antibody	I	NCT01307267
PF-05082566 and pembrolizumab	4-1BB/CD137 antibody + PD1 antibody, respectively	I	NCT02179918
Tremelimumab and MEDI4736	Anti-CTLA4 and anti-PDL1, respectively	I	NCT01975831
MEDI6383	OX40 antibody	I	NCT02221960
MEDI0680 (AMP-514)	PD1 antibody	I	NCT02013804
MEDI0680 (AMP-514) + MEDI4736	PD1 antibody + OX40 antibody, respectively	I	NCT0211833
MPDL3280A	PDL1 antibody	I	NCT01375842, NCT01633970
MSB0010718C	PDL1 antibody	I	NCT01772004
Varlilumab (CDX-1127) and nivolumab	Anti-CD27 mAb that stimulates T-cell receptors	I	NCT02219724
BMS-986016	LAG3 antibody; trials done ± nivolumab (anti-PD1)	I	NCT01968109
Lirilumab	KIR antibody; trials done ± nivolumab (anti-PD1)	I	NCT01714739
TRX518	GITR antibody	I	NCT01239134
MK-4166	GITR antibody	I	NCT02132754

**Abbreviations:** CRCs, colorectal cancers; mAb, monoclonal antibody.

The first FDA-approved immune-checkpoint inhibitor was ipilimumab (anti-CTLA4; Yervoy) for advanced melanoma. In 2014, the FDA granted accelerated approval to pembrolizumab (PD1 inhibitor) for treatment of advanced melanoma. Since the approval of ipilimumab in 2011, another PD1 inhibitor, Opdivo (nivolumab), has been approved by the FDA. Unfortunately, immunotherapy with these antibodies has been disappointing in unselected CRC patients. It is possible that this treatment may be beneficial in selected CRC patients based on predictive biomarkers. A clinical trial with an anti-PD1 mAb is currently in progress in metastatic CRC patients with high microsatellite-instability tumors (NCT01876511). In the case of tremelimumab (anti-CTLA4), one patient showed a partial response, and 21 of 47 refractory metastatic CRC patients (45%) lived  $\geq 180$  days after enrollment.<sup>181</sup> In another study, a patient with refractory CRC treated with nivolumab experienced a complete response for more than 3 years. Further analysis found that the tumors were high in microsatellite instability and were associated with infiltrating macrophages and lymphocytes expressing PDL1.<sup>182,183</sup>

Pembrolizumab (MK-3475) is currently in a Phase II trial for patients with microsatellite-unstable tumors (NCT01876511). Preclinical studies have shown that synergistic antitumor activity can be achieved with a combination of PD1 pathway inhibitors plus inhibitors of other immune checkpoints expressed on T cells, such as *LAG3* and *TIM3*. BMS986016, an antibody against *LAG3*, is in a Phase I clinical trial with or without nivolumab (anti-PD1) in patients with solid tumors (NCT01968109).

The combination of anti-PDL1 therapy with the anti-CTLA4 therapy tremelimumab across multiple tumor types is also in progress. Other current studies include Phase III clinical trials testing the PDL1 inhibitor MPDL3280A in non-small-cell lung cancer (NCT02366143) and MEDI4736 (anti-PDL1 antibody; NCT02227667), a Phase II trial testing the PDL1 inhibitor MSB0010718 (avelumab) in metastatic Merkel cell carcinoma, and Phase I clinical trials for solid tumors. Encouraging results with MPDL3280 and bevacizumab and/or chemotherapy combination were observed in metastatic CRC.<sup>184</sup>

Other antibodies to costimulatory molecules used to enhance T-cell function include urelumab or PF-05082566 (anti-41BB/CD137), varlilumab (anti-CD27), and MOXR0916 (anti-OX40). Phase I trials with urelumab (NCT01471210), PF-05082566 (NCT01307267), PF-05082566 and pembrolizumab (NCT02179918), varlilumab (NCT01460134), varlilumab and nivolumab (NCT02335918), and MOXR0916 are underway.

## Enhancing the function of cells in the innate immune system

A second approach is to enhance the function of innate cells by either engaging cetuximab in complement-mediated cytotoxicity or antibody-dependent cellular cytotoxicity or inducing phagocytosis of cancer cells with anti-CD47.

A Phase II study of Imprime PGG and cetuximab combination in metastatic CRC patients with *KRAS* mutation was conducted.<sup>185</sup> Imprime is a  $\beta$ -glucan polymer that binds to CR3.<sup>186</sup> Cetuximab activates the complement, resulting in production of iC3b, which opsonizes tumor cells. Imprime PGG primes neutrophils and macrophages through a CR3-dependent mechanism to exert killing activity against tumor cells that have been opsonized with complement following targeting with therapeutic mAbs, such as cetuximab.

Preclinical studies have shown that antibody-dependent cellular cytotoxicity by natural killer cells kills EGFR-expressing cancer cell lines following incubation with urelumab (anti-41BB/anti-CD137) in combination with cetuximab.<sup>187</sup> This provided the rationale for studying cetuximab in combination with anti-CD137 (NCT02110082).

Phagocytosis of tumor cells by macrophages is prevented, due to the interaction between CD47 on tumor cells interacting with the ligand SIRP $\alpha$  on macrophages. Preclinical studies have shown that blockade of CD47 with anti-CD47 induces macrophage phagocytosis of tumor cells, including breast, colon, prostate and bladder cancers.<sup>188</sup> The treatment can also prime an effective antitumor T-cell immune response.<sup>189</sup> A Phase I clinical trial with anti-CD47 is underway (NCT02096770).

## Targeting tumor-associated macrophages

Cells in the tumor microenvironment communicate with each other through a complex network of cytokines and growth factors. Some of these cells, such as the macrophages and fibroblasts, enhance tumor progression by modulating angiogenesis and immunosuppression by providing growth factors and other signaling molecules that aid in tumor survival and progression to metastasis. Classically activated macrophages, also referred to as M1 macrophages can kill tumors during the early steps of carcinogenesis. Dynamic changes in the tumor microenvironment occur during the transition from early transformation to advanced tumor stages that gradually drive the switch from M1 to M2 macrophages. Tumor-associated macrophages usually exhibit the M2 phenotype, secreting immunosuppressive cytokines, such as IL10, CCL17, and CCL22.<sup>190</sup> Tumor-associated macrophages represent a dominant myeloid population in many tumors, and their accumula-

tion correlates with poor prognosis.<sup>191</sup> Therefore, they would appear to be rational therapeutic targets in cancer therapy.

One potential strategy is to deplete M2 macrophages. This can be achieved with CSF1R inhibitors. Increased infiltration of cytotoxic T lymphocytes and reduced survival of M2 macrophages were observed in tumors of patients treated with PLX3397 (a multikinase inhibitor targeting CSF1R kinase and c-Kit).<sup>192</sup> Other CSF1R inhibitors, such as BLZ945 and RG7155 (humanized anti-CSF1R antibody), have shown antitumor effects in preclinical models.<sup>193</sup> However, their efficacy in human trials is yet to be determined.

Another approach to modulate the immune system is to use chemokine-receptor antagonists. Welford et al<sup>194</sup> showed that a CXCR4 antagonist (AMD3100) inhibited recruitment of Tie2-expressing macrophages, resulting in increased efficacy of VTAs in a mouse model. A Phase I clinical trial with folinic acid, fluorouracil, irinotecan, oxaliplatin (FOLFIRINOX) plus PF-04136309, a CCR2 chemokine-receptor antagonist that blocks macrophage recruitment and is associated with anti-inflammatory responses, had partial responses in 14 of 29 patients: 14 had stable disease, and only one had progressive disease.<sup>195</sup>

Radiation has been traditionally used to kill tumor cells directly, but has also been shown to have immunomodulatory effects.<sup>196</sup> Ionizing radiation results in immunogenic cell death,<sup>197</sup> releasing endogenous adjuvants that facilitate dendritic cell maturation, uptake of antigens by dendritic cells, and cross-priming of cytotoxic T lymphocytes.<sup>198</sup> In recent years, efforts have been made to augment the antitumor effect of radiation by combining it with immunotherapy.<sup>199</sup> Preclinical trials have shown that CTLA4 blockade acts synergistically with radiotherapy to induce an abscopal response to radiotherapy in murine models of breast cancer and colon cancer.<sup>200</sup> This approach has great potential, and will be worthy of further investigation.

## Challenges in clinical development of targeted and immunobased therapies

### Predictive biomarker development

Advances in DNA-sequencing technologies have led to a comprehensive analysis of exome sequences, DNA copy number, and epigenetic or transcriptomic dysregulation in CRC.<sup>108</sup> The enormous efforts of many researchers have been summarized in a review.<sup>201</sup> These genetic alterations could serve as potential predictive biomarkers of response when selecting patients for targeted therapies. This is exemplified by *KRAS* mutations in selecting patients for EGFR-inhibitor

treatment, such as cetuximab. The mutational profile of tumors, therefore, is important, and they are increasingly incorporated into clinical trials.

In the case of immune-checkpoint inhibitors, predictive biomarker development is still in progress. So far, studies have indicated that patients with tumors high in microsatellite instability and infiltrating macrophages and lymphocytes expressing PDL1 have shown beneficial response to nivolumab.<sup>182,183</sup>

## Existence of CRC subtypes

Molecular classification has categorized CRC into three groups, characterized by chromosomal instability, microsatellite instability, and CpG-island hypermethylation phenotype, respectively. More recently, a new classification of CRC subtypes based on expression profiling has emerged. These new subtypes were found to be associated with treatment outcomes. Patients with goblet and stem-like subtype tumors were found to respond poorly to cetuximab,<sup>202</sup> and those with a mesenchymal-like subtype benefited from MET inhibitors. In another study, CRC tumors were classed into five subtypes that displayed significant differences in clinical and molecular characteristics.<sup>203</sup> In one of these subtypes, there was upregulation of Aurora kinase A, suggesting that these patients could potentially benefit from treatment with inhibitors of Aurora kinase A.

CRC tumors differ depending on their location along the length of the bowel, and thus rectal and colon cancers not only reside in different anatomic locations but also differ in their embryonic origins and metastatic patterns. The proximal part of the colon up to the splenic flexure originates from the embryonic midgut, while the distal colon and rectum originate from the hindgut. The metastatic site of CRC is primarily dictated by its venous blood drainage: the large bowel drains into the portal system, resulting in liver metastases, while the inferior rectal vein drains into the vena cava, resulting in more frequent lung metastases of rectal tumors than from colon tumors.<sup>204</sup>

All the molecular subtypes reported herein are found throughout the length of the colorectal tract, but their frequency differs significantly based on location, and thus microsatellite-instability tumors are found mostly in the proximal colon.<sup>205</sup> Familial CRCs also show significant association with anatomical locations, so familial adenomatous polyposis syndrome develops in the distal colon in the majority of cases (60%), followed by the rectum (25%), in contrast to the Lynch syndrome (hereditary nonpolyposis CRC), which occurs in the proximal column in 55% of cases and only 15% in the rectum.<sup>206</sup> Mutational and epigenetic differences, including gene methylation between the three main

locations, are also significant.<sup>207</sup> Patients with metastatic CRC arising from distal primary tumors have a better prognosis for first-line chemotherapy alone or in combination with bevacizumab treatment.<sup>208</sup>

Due to their anatomic location, rectal cancers are treated differently to colon cancers, and invariably require neoadjuvant radiotherapy or radiochemotherapy to reduce the tumor prior to resection. These treatments have the potential to alter recurrent tumor characteristics locally or in metastases, impacting further disease management. As outlined in the immunobased therapies section, radiotherapy is known to kill tumor cells in an immunogenic manner and to induce systemic antitumor immune responses that may target and eliminate distant untreated micrometastases. In the clinic, however, additional enhancement of the immune system is required, such as that covered in the previous section. Chemoradiotherapy of rectal tumors before resection was shown to reduce immune-cell levels in the tumor, peritumoral tissues, and circulation compared to these tissues from patients who underwent surgery alone;<sup>209</sup> however, coadministration of PSK, an immunostimulatory protein-bound polysaccharide from the basidiomycete *Coriolus versicolor*,<sup>210</sup> during chemoradiotherapy restored immune-cell levels.<sup>211</sup> Among its other immunostimulatory effects, PSK was shown to induce maturation of antigen-presenting dendritic cells.<sup>212</sup> Another study found high levels of CXCR4 and CXCL12 expression in primary rectal tumors of patients presenting with metastatic disease. Treatment with radiochemotherapy and bevacizumab further upregulated CXCL12 expression.<sup>213</sup> The CXCL12 chemokine attracts immunosuppressive/proangiogenic myeloid-derived suppressor cells and tumor-associated macrophages expressing the CXCR4 receptor into the tumor microenvironment, suggesting that inclusion of inhibitors or receptor antagonists of the CXCL12–CXCR4 axis would improve treatment outcome. A relevant study combining radiotherapy with tadalafil, a small-molecule inhibitor of myeloid-derived suppressor cells function, is ongoing for patients with locally advanced and borderline resectable pancreatic cancer (NCT01903083).

These studies suggest that expression profiling can further enhance our knowledge on the biological reasons for responsiveness or unresponsiveness to targeted therapies. This information can be used for the development of predictive markers using less complex methods, which can be further validated and potentially adopted in routine clinical diagnostics.

## Resistance due to cancer stem cells

The cancer stem cell theory states that cancer stem cells are distinct from cells that form the bulk of a tumor in that

they can self-renew and differentiate to produce progenitor cells, the way traditional stem cells do. This property enables cancer stem cells to self-renew and generate progenies that can differentiate into the bulk of the proliferating cancer cells within the tumor,<sup>214</sup> and like traditional stem cells they are more resistant to chemotherapy. Therefore, therapies specifically targeting cancer stem cells are essential in combination with other treatments to kill the tumor.

Preclinical research with a small molecule, PTC-209, which targets the self-renewal regulator BMI1, appears promising, as it abrogated the tumorigenic capacity of colon cancer stem cells.<sup>215</sup> Inhibitors of signaling pathways, such as Notch, Wnt, and Hh, have also been shown to kill these putative cancer stem cells. One example is demonstrated by the  $\gamma$ -secretase inhibitor PF-03084014 plus irinotecan, which resulted in reduced tumor recurrence in a CRC-explant model. The bulk of these effects were seen in ALDH<sup>+</sup> tumor cells, which is a subpopulation enriched for cancer-initiating cell activity.<sup>150</sup> Selective  $\beta$ -catenin-binding protein antagonists, such as PRI-724, have also been shown to eliminate cancer stem cells by forced differentiation.<sup>216</sup>

Hh-signaling pathway activation has also been shown to play a role in the maintenance of cancer stem cell phenotype. Preclinical studies have shown that inhibitors of Hh-signaling pathways, such as Smo antagonists, can be used to target cancer stem cells.<sup>217</sup> Therefore, elimination of cancer stem cells has been demonstrated with an inhibitor of the self-renewal regulator BMI1 and inhibitors of the Wnt-, Notch-, and Hh-signaling pathways.

## Future directions

### Recent progress for development of an in silico prescription strategy

As of September 2013, a number of next-generation sequencing (NGS) studies in CRC have been reported.<sup>108,218–220</sup> NGS panels have been developed to check tumors for mutations, and currently panels of five to 500 mutations are available for evaluation. Interestingly, a recent report on in silico analysis of genetic mutations for selecting the large pool of previously discovered targeted drugs has potential for prescription strategy beyond targeting EGFR and VEGF systems.<sup>221</sup> The in silico prescription strategy requires three steps. Firstly, NGS data need to be analyzed to discover actionable driver events using DriverDB.<sup>222</sup> Secondly, information on therapeutic agents needs to be collected from the Drivers Actionability Database. Lastly, the data of both databases will need to be combined to obtain a list of actionable targets with their matching targeted therapeutic agent.

The application of NGS has not only provided a wide spectrum of mutational profiles but also additional information, such as splice variants and novel fusion genes. A novel fusion protein found in three of 97 CRC patients is VTIIA–TCF7L2, which offers a potential new therapeutic target.<sup>218</sup> Another example is novel fusion genes encoding R-spondin fusion proteins, which have the ability to potentiate Wnt signaling and thereby function as stem cell growth factors.<sup>219</sup>

Some of the complicated issues regarding NGS include the fact that the majority of mutations will not provide clinical actionable information, the difficulties of interpreting substantial amounts of genomic data, timing of the tests, and access to treatment options. Integration of the data to enable a better understanding of the biology in order to select appropriate targeted drugs is essential for further progress in this field. This will require a lot of effort and money.

## Combination of targeted therapies and silencing of genes by siRNA and microRNAs

RNA-interference strategies appear to be an attractive approach to inhibit expression of oncogenes. Indeed, small interfering RNA (siRNA) has emerged as a major tool to silence gene expression.<sup>223</sup> However, clinical trials on the delivery of siRNA in humans are scarce,<sup>224</sup> and the development of siRNA as a therapeutic tool has been hindered, due to problems with delivery. A recent publication showed that siRNA against Ras physically coupled to cetuximab exerted antitumor activity on colon cancer mouse xenografts.<sup>225</sup> Another potential treatment strategy is to use microRNAs to repress or degrade targeted messenger RNAs.<sup>226</sup> It has been demonstrated that miR-4689 delivered into cells was able to induce apoptosis of *KRAS*-mutant colon cancer cell lines and also exert antitumor activity on mouse xenografts when the microRNA complexes were administered intravenously.<sup>227</sup> The miR-4689 targeted both Raf/MEK/ERK and PI3K/Akt pathways through direct inhibition of K-Ras and Akt1. Lastly, genome-editing technologies using zinc-finger nucleases, transcription-like effector nucleases and clustered regularly interspaced short palindromic repeat-associated nuclease 9<sup>228</sup> to remove deleterious “driver mutations” or to insert “protective mutations” may present novel strategies for cancer therapy in the future.

## Conclusion

Although there are many targeted drugs in clinical trials, many have produced disappointing results. However, with further discovery of novel driver genes through NGS technology, new drug development, and application of in silico analysis, we will

be moving toward developing a personalized prescription for cancer treatment. New developments in the use of immune-checkpoint inhibitors and immunotherapy will be seen in the coming years. Further research is needed to elucidate the contribution of the tumor microenvironment, the immune response, and the host–drug response in the development of tumor resistance, in order to achieve an effective treatment strategy. These new discoveries will contribute to our increased understanding of the heterogeneity of most tumors, events in the tumor microenvironment, and new gene and protein signatures that can be translated into clinically meaningful improvements. It is without doubt that application of bioinformatics will be required to link molecular signatures as predictive biomarkers for drug response to overcome the current problems and enable individualized drug therapy for cancer patients. Advances in RNA interference, noncoding RNA, and genome editing will pave the way for an exciting future in gaining new insights into cancer biology and the development of new therapeutic strategies in cancer therapy.

## Disclosure

The authors report no conflicts of interest in this work.

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