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Therapeutic implications of resistance to molecular therapies in metastatic colorectal cancer

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SUMMARY

Metastatic colorectal cancer (mCRC) patients carrying KRAS mutated tumors do not benefit from epidermal growth factor receptor (EGFR)-targeted cetuximab- or panitumumab-based therapies. Indeed, the mutational status of *KRAS* is currently a validated predictive biomarker employed to select mCRC patients for EGFR targeted drugs. When patients fail standard 5-fluorouracil-, oxaliplatin-, irinotecan- and bevacizumab-based therapies, EGFR-targeted salvage therapy can be prescribed only for those individuals with *KRAS* wild-type cancer. Thus, clinicians are now facing the urgent issue of better understanding the biology of *KRAS* mutant disease, in order to devise novel effective therapies in such defined genetic setting. In addition to *KRAS*, recent data point out that *BRAF* and *PIK3CA* exon 20 mutations hamper response to EGFR-targeted treatment in mCRC, potentially excluding from treatment also patients with these molecular alterations in their tumor. This review will focus on current knowledge regarding the molecular landscape of mCRC including and beyond KRAS, and will summarize novel rationally-developed combinatorial regimens that are being evaluated in early clinical trials.

Background

The introduction of *KRAS* testing as a diagnostic tool to select patients for epidermal growth factor receptor (EGFR)-targeted cetuximab- or panitumumab-based therapies for metastatic colorectal cancer (mCRC) has been validated and is regarded as one of the most important recent advances in the field of personalized cancer medicine.[1] The oncogene *KRAS* is indeed the most commonly mutated gene in various human cancers and it has been demonstrated that its constitutive activation in mCRC can bypass the EGFR-driven signalling cascade and impair the clinical efficacy of EGFR-targeted monoclonal antibodies.[2] *KRAS* testing has rapidly led to an improvement in the therapeutic index of these drugs, excluding from treatment patients harbouring mutations in the tumor, who do not achieve clinical benefit from these targeted therapeutics, as recommended by the American Society of Clinical Oncology (ASCO).[3] Thus, clinicians are now facing the emerging issue of better understanding the biology of the *KRAS* mutant disease, because the unfeasibility of EGFR-targeted salvage therapy leaves an unmet need for treatment options in those patients who fail standard 5-fluorouracil-, oxaliplatin-, irinotecan- and bevacizumab-based therapies.

In a recent paper by De Roock et al.,[4] 649 tumour DNA samples from chemotherapy-refractory mCRC patients treated with cetuximab plus chemotherapy were gathered from 11 centres in seven European countries, and investigators show that also *BRAF, NRAS*, and *PIK3CA* exon 20 mutations were significantly associated with a low response rate, confirming findings from previous patients' cohort analyses.[5,6] This suggests that also patients harbouring these molecular alterations should be excluded from EGFR-targeted treatment with cetuximab, thus lacking effective third-line treatment strategies. At present, each of these markers (*KRAS, BRAF, PIK3CA*) has been mainly assessed as a single event, often in retrospective analyses and patients series, but these molecular alterations display overlapping pattern of occurrence, thus adding complexity for drawing an algorithm suitable for clinical decision-making. For this reason, last-generation studies by our group and others nowadays include comprehensive integrated analysis of the entire oncogenic pathway triggered by the EGFR, with the aim of enhancing the prediction ability of the markers individually used.[7]

There is an urgent need to develop effective salvage therapies for patients with primary refractoriness to EGFR targeted monoclonal antibodies (i.e. those affected by *KRAS*/*BRAF*/*PIK3CA* mutated tumors), as well for those who develop resistance over prolonged

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treatment. This review will focus on agents targeting the KRAS pathway, given its pivotal role in the biology of CRC and response to anti-EGFR therapies. Additionally, we will outline clinical trials with drugs directed at molecular targets that might be synthetically lethal with concomitant EGFR inhibition.

Targeting molecular effectors downstream of EGFR interacting with RAS that could preclude responsiveness to cetuximab or panitumumab

BRAF

BRAF is a cytoplasmatic serine/threonine kinase directly interacting with RAS, which regulates its activity starting a cytoplasmic phosphorylation cascade which leads to the activation of transcription factors controlling cell growth, differentiation and apoptosis (the ERK signalling pathway).[8] The *BRAF*^{V600E} mutation, a thymine to adenine transversion mutation, resulting in the substitution of valine with glutamate, appears in 4–15% of CRC.[4,7] Importantly, *KRAS* and *BRAF* mutations are known to be mutually exclusive in CRC,[9] and, as for *KRAS*, there is a high concordance of *BRAF* mutations in primary CRC and related metastatic sites.[10] The first study assessing the role of the *BRAF*^{V600E} mutation as a predictive molecular factor to EGFR-targeted therapy was performed by our group in a cohort of 113 tumors from patients who received panitumumab/cetuximab in second or subsequent lines of treatment.[5] Results from this retrospective analysis showed that, among *KRAS* wild-type patients, those whose tumors displayed the *BRAF*^{V600E} mutation (14%) did not respond to EGFR inhibition and had statistically significantly shorter progression-free survival (PFS) and overall survival than patients whose tumors carried wild-type *BRAF*. In the same article, we also demonstrated that introduction of the *BRAF*^{V600E} allele could confer resistance to either cetuximab or panitumumab in wild-type *BRAF* CRC cells. Subsequently, Loupakis et al. performed a retrospective analysis among 87 irinotecan refractory patients, treated with anti-EGFR therapy.[11] They found that *BRAF* was mutated in 13 cases (15%): none of the patients bearing *BRAF* mutation responded to the treatment, in comparison with 24 (32%) of 74 patients with *BRAF* wild-type disease ($p = 0.016$). *BRAF* mutation was also associated with a trend towards shorter *PFS* and with significantly shorter overall survival. In the recent wide retrospective cohort analysis of chemorefractory patients from a European Consortium ($n = 761$), De Roock et al. reported 4.7% *BRAF* mutations: among *KRAS* wild-type patients treated with cetuximab a significantly lower response rate, shorter PFS and overall survival than with wild-type tumors were observed, and this effect was confirmed in multivariate analyses performed using the mutation status of *KRAS, PIK3CA* exon 20, *PIK3CA* exon 9, *BRAF*, and *NRAS*, and age, sex, number of previous chemotherapy lines, and European centre as covariates.[4]

In summary, data from these[4,5,11] and other studies[7] clearly show that, in the CRC chemorefractory setting, *BRAF* mutations are predictive of resistance to EGFR-targeted monoclonal antibodies. Since RAF is an important effector downstream of RAS in the ERK signalling pathway, targeting this effector could be also viewed as an effective strategy for treating *KRAS* or *BRAF* mutated tumors. We showed indeed that one of the first inhibitors of RAF activity, sorafenib, may restore sensitivity to EGFR inhibitors in *BRAF*-mutated CRC cell lines[5] and, consequently, combined sorafenib and cetuximab therapy is undergoing clinical evaluation in mCRC in a National Cancer Institute-sponsored trial (ClinicalTrials.gov Identifier: NCT00343772, Table 1). Nevertheless, it should be taken into account that sorafenib is a multikinase inhibitor which does not work primarily as a RAF inhibitor, but rather as an inhibitor of angiogenesis by inhibiting VEGFR-1, -2, -3, and PDGFR,[12] and thus better results would have been expected by more selective RAF inhibitors such as PLX4032, that showed pronounced activity in *BRAF*-mutant melanoma patients. Unexpectedly, in a recent report from Kopetz et al., evaluating 19 mCRC *BRAF*^{V600E} patients treated with PLX4032, only a modest clinical activity was observed (1 confirmed partial response, 4 minor responses with ≥10% shrinkage and 5 mixed responses), suggesting that the biology of BRAF activation in patients with mCRC is clearly more heterogeneous than in melanoma, as evidenced in those patients with a mixed response.[13] Interestingly, Hatzivassiliou et al. recently demonstrated that ATP-competitive RAF inhibitors, such as GDC-0879 and PLX4720, possess two opposing mechanisms of action depending on the cellular context, i.e., in *BRAF*^{V600E} tumours, RAF inhibitors effectively block the MAPK signalling pathway and decrease tumour growth, while in *KRAS* mutant and *KRAS/BRAF* wild-type tumours, RAF inhibitors activate the RAF-MEK-ERK pathway in a RAS-dependent manner, thus enhancing tumour growth in xenograft models.[14] Conversely, the MEK inhibitor PD0325901 inhibits proliferation of *BRAF*^{V600E}, *KRAS/BRAF* wild-type and *KRAS* mutant cancer cells.[14] Similar findings come also from the study by Poulikakos et al., showing that ATP-competitive RAF inhibitors inhibit ERK signalling in cells with mutant *BRAF*, but paradoxically enhance signalling in cells with wild-type *BRAF* by drug-mediated transactivation of RAF dimers.[15]

From a clinical standpoint, these preclinical results indicate the working hypothesis that RAF inhibitors may be used in mCRC in which *BRAF* only is mutated, whereas MEK inhibitors could be effective in a wider range of conditions: *BRAF*^{V600E}, *KRAS/BRAF* wild-type and *KRAS* mutant tumors. It remains to be shown whether concomitant blockade of EGFR and BRAF or MEK would result in increased clinical efficay in BRAF/KRAS mutant tumors. Table 1 shows selected ongoing studies with EGFR-directed monoclonal antibodies in combination with other targeted agents, including BRAF inhibitors.

PIK3CA and PTEN

In addition to RAS and RAF, the EGFR also activates the PI3K signaling pathway, which in turn can be oncogenically deregulated either by activating mutations in the *PIK3CA* p110 subunit or by inactivation of the PTEN phosphatase. Importantly, there is an interaction between RAS and PI3K, since the PI3K signaling pathway can be activated both by EGFR as well as by RAS itself.[16] The role of deregulated *PIK3CA*/PTEN signaling on the response to targeted therapy has been investigated in breast, glioblastoma and also mCRC. *PIK3CA* mutations occur in approximately 10–18% of CRC patients, principally located in exon 9 and 20,[4,6,17] whereas loss of PTEN expression by immunohistochemistry (IHC) is reported in 19–42%.[18,19] *In vitro* studies in various CRC cell lines have found that activating *PIK3CA* mutations or loss of PTEN expression appear to confer resistance to cetuximab: cell lines carrying mutations in *PIK3CA*, or displaying loss of PTEN, with concomitant mutations in *RAS* or *BRAF* exhibit the greatest resistance to cetuximab.[20] In the clinical setting, we found that in a cohort of 110 patients *PIK3CA* mutations and PTEN loss were statistically significantly associated with lack of response to panitumumab (0/15 patients, $p = 0.038$) or cetuximab (1/32 patients, $p = 0.001$) treatment.[6] In the same study, *PIK3CA* mutations and/or loss of PTEN expression were negatively associated with PFS, and loss of PTEN expression was also linked with poorer overall survival ($p = 0.005$). This negative association with PFS was also noted in a study by Souglakos et al.,[21] where among 92 patients treated using chemotherapy and cetuximab as salvage therapy, *PIK3CA* mutations predicted reduced PFS (2.5 vs 3.9 months, HR 2.1, 95% CI 1.2–3.9). In contrast, Prenen et al. reported in a series of 200 mCRC patients that 23 (12%) carried a *PIK3CA* mutation and 5 of these (22%) were found in responders.[22] This means that 5 of 39 responders (13%) and 18

Table 1
Selected clinical trials evaluating the combination of EGFR-directed therapeutics with other targeted agents. Trials were retrieved from the U.S. National Institutes of Health service *ClinicalTrials.gov* and updated as of July, 30th

Anti-EGFR agent	Drug 2	Drug 3	Phase	NCT	Notes
Cetuximab	E7820		II	NCT00309179	E7820 is an oral antiangiogenic sulfonamide that inhibits alpha-2 integrin. 1 partial response observed in *KRAS* mutant mCRC
	Erlotinib		II	NCT00784667	Among 50 patients, RR of 52% in patients who were both *KRAS*/*BRAF* wild-type. No responses in 11 patients with *KRAS* mutations and in 8 with *BRAF* mutation
	Sorafenib		II	NCT00343772	
	Temsirolimus		I	NCT00593060	
	Irinotecan	Everolimus	I	NCT00522665	
	Lenalidomide		II	NCT01032291	In *KRAS* mutant mCRC
	Irinotecan	EMD525797	I/II	NCT01008475	EMD525797 is a monoclonal antibody specific for the human alpha-V integrin subunit. It inhibits human endothelial adhesion to vitronectin
	Irinotecan	ARQ 197	I/II	NCT01075048	ARQ 197 is a cMET inhibitor
	FOLFIRI	IMO-2055	1b	NCT00719199	IMO-2055 is a synthetic oligonucleotide with immunopotentiating activity (TLR9 agonist)
	ZD6474		I	NCT00436072	ZD6474 is a dual VEGFR and EGFR inhibitor
	Pertuzumab		I/II	NCT00551421	Pertuzumab is a humanized anti-HER-2 monoclonal antibody
	Irinotecan	Dalotuzumab (MK-0646)	I	NCT00925015; NCT00614393	Dalotuzumab is a humanized anti-IGF-1R monoclonal antibody
	Irinotecan	Sunitinib	I	NCT00361244	
	Irinotecan	Pazopanib	I	NCT00540943	Pazopanib is a multi-targeted tyrosine kinase inhibitor of VEGFR, PDGFR and c-kit
	Dasatinib		I	NCT00835679	
	BMS-908662 (XL281)		I	NCT01086267	BMS-908662 (XL281) is an oral BRAF inhibitor. In *KRAS* or *BRAF* mutant mCRC
	FOLFIRI	PRO95780		NCT00497497	PRO95780 is a human monoclonal antibody activating pro-apoptotic Death Receptor 5 (DR5)
	BMS-754807		I	NCT00908024	BMS-754807 is an oral IGF-1R tyrosine kinase inhibitor
	Bortezomib		I	NCT00622674	
	Brivanib		I	NCT00207051	Brivanib alaninate is an oral dual inhibitor of VEGFR and FGFR
Panitumumab	FOLFOX/FOLFIRI	AMG 706 (Motesanib)	Ib/2	NCT00101894	Motesanib is an oral tyrosine kinase inhibitor of VEGFR, PDFGR, and c-kit
	AMG 102		I/II	NCT00788957	AMG 102 is a human anti-HGF/SF monoclonal antibody
	AMG 479		I/II	NCT00788957	AMG 479 is a human anti-IGF-1R monoclonal antibody
	Imatinib		I/II	NCT00867334	Patients are prescreened for c-kit/PDGFR activated pathways using a proteomic-based assay
	AMG 479	Everolimus	I	NCT01061788	
	AMG 706 (Motesanib)		I	NCT00101894	Motesanib is an oral tyrosine kinase inhibitor of VEGFR, PDFGR, and c-kit
	Decitabine		I/II	NCT00879385	in *KRAS* wild-type mCRC
	Simvastatin		II	NCT01110785	in *KRAS* mutant mCRC
	Conatumumab		I/II	NCT00630786	Conatumumab is a pro-apoptotic TRAIL receptor-2 agonist

NCT: ClinicalTrials.gov Identifier; mCRC: metastatic colorectal cancer.

of 160 non-responders (11%) carried a *PIK3CA* mutation, thus not supporting a significant association between *PIK3CA* mutations and lack of response to cetuximab (p = 0.781). The median PFS and overall survival did also not differ significantly between *PIK3CA* mutant and wild-type patients. Finally, the large dataset by the European Consortium showed that among 356 *KRAS* wild-type chemorefractory tumors treated with cetuximab, patients with mutant *PIK3CA* as a whole had a significantly lower response rate compared with carriers of wild-type *PIK3CA*, (17.7% [6/34] *vs* 37.7% [115/305]; OR 0.35, 95% CI 0.13–0.83; p = 0.015). Notably, there was no significant difference in PFS and overall survival (median PFS 18 vs 24 weeks, HR 1.30, 95% CI 0.91–1.86; p = 0.17; and median overall survival 39 vs 51 weeks; HR 1.41, 0.96–2.06; p = 0.09).[4] However, when compared with *PIK3CA* wild-type, *PIK3CA* exon 20 mutations had a negative effect on objective response (0.0% [0/9] vs 36.8% [121/329], Fisher's exact test estimated OR 0.00, 95% CI 0.00–0.89; p = 0.029), disease control (33.3% [3/9] vs 76.0% [250/329]; OR 0.158, 0.0327–0.613; p = 0.0078), PFS (median 11.5 vs 24 weeks, HR 2.52, 1.33–4.78; p = 0.013), and overall survival (median 34 vs 51 weeks; HR 3.29, 1.60–6.74; p = 0.0057), whereas *PIK3CA* exon 9 mutations had no significant effect on response rate, median PFS, and median overall survival.

Taken together, these data highlight the role of *PIK3CA* exon 20 mutations in predicting resistance to cetuximab and panitumumab, although this association should be confirmed in prospective trials. The different impact on clinical outcome exerted by exon 9 and exon 20 mutations is explained by *in vitro* studies, demonstrating that mutations located in different hotspots give rise to different

biochemical and oncogenic properties and are differently activated by RAS.[23] Conflicting results from previous published works[6,22] could therefore be explained by the heterogeneity of patients series in terms of the distribution of mutations in the two different exons.

Pharmacological inhibition of PI3K for cancer treatment is a strategy currently under investigation in several phase I and II trials. Given the frequency and role of oncogenic *PIK3CA* mutations in mCRC above described, it would be rationale to target this pathway in the *KRAS* wild-type population. Indeed, the AKT inhibitor MK-2206 is currently ongoing phase II testing in mCRC chemorefractory patients with *KRAS* wild-type, *PIK3CA*-mutated, mCRC [ClinicalTrials.gov Identifier: NCT01186705] or together with EGFR-targeted monoclonal antibodies to circumvent resistance. In light of recent data,[4] the latter approach should hypothetically be restricted to the rare subset of patients harboring exon 20 mutations. On the other hand, because of the interaction between RAS and PI3K,[16] oncogenic *KRAS* itself can prompt cancer cells for escaping pharmacological MEK blockade by activating feedback loop between RAF-MEK-ERK and PI3K pathways.[24] Consequently, in a breast cancer preclinical models, dual inhibition with MEK and PI3K inhibitors result in a synergistic tumor growth inhibition.[25] Therefore, concomitant PI3K and MEK inhibition appear a promising strategy for *KRAS* mutant-mCRCs, potentially overcoming resistance conferred by compensatory cross-talk between pathways. A phase I clinical trial applying this approach with the PI3K inhibitor BKM120 given in combination with the MEK inhibitor GSK1120212 is currently ongoing in patients with advanced solid tumors selected for *KRAS*/*BRAF* mutations [ClinicalTrials.gov Identifier: NCT01155453].

Targeting other cell-surface receptors

A different pharmacological strategy to treat *KRAS* mutant tumors is represented by targeting receptors tyrosine kinase other than EGFR that contribute to enhanced cell survival and proliferation. The type 1 insulin-like growth factor receptor (IGF-1R) is a member of a family of transmembrane tyrosine kinases that includes the insulin receptor and the insulin receptor-related receptor. The IGF-1R signaling pathway is an important pathway in different types of cancers including CRC[26] and include transduction of the IGF signal by the mitogen-activated protein kinase and PI3K/Akt pathways.[27] Recent evidence suggested a role for IGF-1R signaling in the acquired resistance to EGFR inhibitors in glioblastoma cells[28] and there is evidence for cross-talk between IGF-1R and EGFR.[29] Basing on these findings and on preclinical data showing that combination treatment of IGF-1R and EGFR kinase inhibitors result in synergy of growth inhibition in CRC cell lines,[29] a phase II study with the anti-IGF-1R monoclonal antibody IMC-A12, either alone or in combination with cetuximab, was performed in patients with cetuximab- or panitumumab-refractory mCRC. In this study,[30] 64 patients were treated (23 patients with IMC-A12 monotherapy, 21 with IMC-A12 plus cetuximab and 20 with IMC-A12 plus cetuximab among patients who had disease control on a prior anti-EGFR monoclonal antibody and wild-type *KRAS* tumors). No antitumor activity was seen in the 23 patients treated with IMC-A12 monotherapy and of the 21 patients treated with the combination with cetuximab, one patient (with *KRAS* wild-type) achieved a partial response, with disease control lasting 6.5 months. No additional antitumor activity was observed in patients treated with IMC-A12 plus cetuximab who showed disease control on a prior anti-EGFR monoclonal antibody and wild-type *KRAS* tumors. These results indicate no meaningful antitumor activity in this setting (overall, 1 response out of 64 patients) and do not suggest further development of the drug in this setting. Nevertheless, it is interesting to note that the one patient responding to the combination of IMC-A12 and cetuximab had a tumor that was wild-type for *KRAS*, *NRAS*, *BRAF*, and *PIK3CA*. The authors concluded that *KRAS* wild-type status may be required (but not sufficient) to confer IGF-1R dependence, thus suggesting that this approach is not appropriate for *KRAS* mutant mCRC. Further studies with anti-IGF-1R agents are ongoing in mCRC, including a phase II biomarker study led at our Institutions with the anti-IGF-1R monoclonal antibody AMG 479. In this study (ClinicalTrials.gov Identifier: NCT00891930), mCRC *KRAS* wild-type patients pretreated with irinotecan- and oxaliplatin- or oxaliplatin-based chemotherapy undergo a baseline tumor biopsy and then receive panitumumab with irinotecan (part 1 of the study); at disease progression patients that have displayed response or stable disease undergo a second tumor biopsy and then proceed to part 2 of the study including treatment with panitumumab in combination with AMG 479 with the aim of overcoming acquired resistance to EGFR-targeted therapy. This study will provide insights about mechanisms of secondary resistance (i.e. potential change in *KRAS* mutation status from wild-type at baseline to mutant at the time of the second biopsy following evidence of acquired resistance to panitumumab and irinotecan) and about the potential role of IGF-1R-targeted therapy in overcoming resistance to panitumumab.

The hepatocyte-growth factor (HGF)-mesenchymal epithelial transition factor (MET) molecular pathway is also well known as an important pathway in cancer development. Moreover, MET-related signal transduction is thought to be involved in the development of resistance to EGFR targeting agents[31] and the combinatorial inhibition of HGF-MET and EGFR is therefore an interesting approach to assess in clinical trials.[32]

In conclusion, RAS plays a central role in EGFR and other receptors tyrosine kinase signaling, thus its constitutive activation could also hamper approaches involving inhibition of IGF-1R and MET pathway. Therefore, in *KRAS* mutant CRC patients, a multi-targeted strategy including combination of both MET or IGF-1R inhibitors together with inhibitors of targets downstream of RAS is probably the best approach. Interestingly, concomitant blockade of IGF-1R and MEK has been shown effective to prevent the occurrence of the EGFR-IGF1R cross-talk and showed preclinical activity in BRAF mutated CRC preclinical models.[33] Therefore, combinatorial clinical studies might be warranted for chemorefractory mutated mCRC.

Conflict of interests

All authors have no conflict of interest to declare.

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