KRAS, *BRAF*, *PIK3CA*, and *PTEN* mutations: implications for targeted therapies in metastatic colorectal cancer

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The discovery of mutant *KRAS* as a predictor of resistance to epidermal growth-factor receptor (EGFR) monoclonal antibodies brought a major change in the treatment of metastatic colorectal cancer. This seminal finding also highlighted our sparse knowledge about key signalling pathways in colorectal tumours. Drugs that inhibit oncogenic alterations such as phospho-MAP2K (also called MEK), phospho-AKT, and mutant B-RAF seem promising as single treatment or when given with EGFR inhibitors. However, our understanding of the precise role these potential drug targets have in colorectal tumours, and the oncogenic dependence that tumours might have on these components, has not progressed at the same rate. As a result, patient selection and prediction of treatment effects remain problematic. We review the role of mutations in genes other than *KRAS* on the efficacy of anti-EGFR therapy, and discuss strategies to target these oncogenic alterations alone or in combination with receptor tyrosine-kinase inhibition.

Introduction

Targeted drugs for the treatment of cancer have rapidly developed. However, our understanding (at the molecular level) of the precise role that potential targets have in tumorigenesis, and the survival dependence that tumours have on these components, has not progressed at the same rate. Therefore, patient selection remains problematic—eg, less than 20% of patients with metastatic colorectal cancer (mCRC) respond to clinically available targeted drugs when used as monotherapy.[1,2] Clearly, individualised treatment is needed. Colorectal cancer is a heterogeneous disease defined by different activating mutations in receptor tyrosine kinases (RTKs), or activating or loss-of-function mutations in downstream components of RTK-activated intracellular pathways, some of which can occur in the same tumour. The efficacy of targeted drugs is therefore linked to the specific molecular alterations in the tumour. The epidermal growth-factor receptor (EGFR) monoclonal antibodies cetuximab and panitumumab are highly effective in a subset of patients with mCRC. In this Review, we describe key components of the EGFR-signalling pathway that are altered in mCRC and the potential therapeutic efficacy of selective molecularly targeted drugs.

Molecular mechanisms of primary resistance to EGFR antibodies

EGFR (also called ERBB1/HER1) is an RTK belonging to the ERBB-family. Cetuximab and panitumumab block ligand-induced EGFR tyrosine-kinase activation, thereby probably preventing downstream activation of phosphatidylinositol 3-kinase (PI3K)/AKT and RAS/MAP2K (also called MEK)/MAPK1/3 (also called ERK2/1) signalling pathways (figure), resulting in inhibition of cellular proliferation and induction of apoptosis.[1] A series of genetic and biological characteristics of colorectal cancer thought to be linked to increased EGFR-dependency have been associated with increased efficacy of EGFR monoclonal antibodies. Increases in EGFR copy number were associated with tumour response to cetuximab and panitumumab,[3–6] and the level of sensitivity to cetuximab was proportional to the level of mRNA expression of two EGFR ligands, epiregulin (EREG) and amphiregulin (AREG).[7–9] These data on potential positive predictors need to be further investigated and validated. A larger body of evidence is available for negative predictors, identifying patients that should not be treated with these drugs. Data suggest that expression of phospho-MAP2K1 and phospho-RPS6K might be associated with shorter progression-free survival in patients with mCRC who are given cetuximab.[10] More comprehensive data suggest that oncogenic mutations in genes encoding key downstream effectors within the EGFR-signalling pathways are responsible for primary intrinsic resistance to EGFR monoclonal antibodies (table 1).[19] We discuss the biology of these genes, the effect of mutant forms on the efficacy of EGFR monoclonal antibodies, and their hypothetical effects on the efficacy of other targeted therapies.

KRAS

KRAS belongs to the *RAS* family of genes (*KRAS*, *NRAS*, and *HRAS*) that encode guanosine-5´-triphosphate (GTP)-binding proteins. KRAS is an important effector of ligand-bound EGFR, mainly but not exclusively signalling through *BRAF* and the MAPK axis. KRAS can also activate PI3K through direct interaction with its catalytic subunit.[20] Around 32–40% of colorectal cancers harbour a *KRAS* mutation.[11,12,21,22] About 85–90% of these mutations occur in codons 12 or 13. The remaining mutations mainly occur in codons 61 (5%) and 146 (5%).[11,12,22] These mutations disable GTPase activity, causing tumour-associated KRAS to accumulate in the active GTP-bound conformation.

Ten retrospective studies (single-group studies and randomised clinical trials, summarised by Allegra and colleagues[11]) confirmed the finding by Lièvre and colleagues[23] that mutant *KRAS* is a predictor of resistance to EGFR monoclonal antibodies. This discovery led to the first practical implementation of personalised medicine in mCRC. All patients with mCRC are now

profiled for seven mutations in *KRAS* codons 12 and 13 before receiving cetuximab or panitumumab.[19] A European consortium study[12] showed that codon-61 mutations had an adverse effect similar to codon-12 mutations, whereas codon-146 mutations did not affect cetuximab efficacy. Codon-146 mutations co-occurred with other *KRAS* mutations—an additional indication that this might not be an important oncogenic codon.[12] In a large retrospective pooled exploratory analysis of chemotherapy-refractory patients, a positive association between KRAS G13D mutations and cetuximab treatment was seen in regard to better overall and progression-free survival. However, prospective randomised trials are needed before conclusions about potential beneficial effects of cetuximab in G13D-mutated chemotherapy-refractory metastatic colorectal cancer should be inferred.[24]

The effect of *KRAS* mutations seen in patients with mCRC who are given EGFR monoclonal antibodies is unlikely to be prognostic (independent of any specific treatment) and more likely to be predictive (attributable to treatment). In a retrospective analysis of the randomised CRYSTAL study[14] in first-line mCRC, a significant association was shown between KRAS mutation status and objective response, progression-free survival, and overall survival. However, the quest for predictive biomarkers continues, since up to 50–65% of patients with *KRAS*-wild-type tumours are resistant to EGFR monoclonal antibodies.[11,12] Other genetic alterations in *KRAS*-wild-type tumours could cause primary resistance. Accumulating evidence suggests that *BRAF*[4,13] and *PIK3CA*[12,15] mutations might affect response to EGFR monoclonal antibodies. It is unclear to what extent the effects of mutant *KRAS* are the same for other RTK-targeted therapies. It is possible that *KRAS*-mutant tumours are not dependent on any RTK upstream component, and therefore will not respond to drugs targeting these RTKs. Alternatively, it might be that *KRAS* mutations confer only part of the survival advantage needed for tumour cells, and additional signals derived from RTK signalling are needed, in which case *KRAS*-mutant tumours will still benefit from RTK inhibition. Moreover, to define colorectal cancer as *KRAS* mutant versus *KRAS* wild-type probably underestimates additional heterogeneity found within both populations. In the mutant *KRAS* population, the ultimate levels of, and dependence on, MAPK1/3 signalling by the tumour cells depend on other features of the tumour. In this regard, the dual specificity phosphatase (DUSP) and sprouty homologue (SPRY) negative-feedback loops (figure) naturally present in the cell can attenuate the MAPK1/3 output in *KRAS*-mutant tumours. Pratilas and colleagues[25] reported that DUSP and SPRY expression were associated with MAP2K dependency of tumours, and similarly, we showed a correlation between DUSP4 expression and sensitivity to cetuximab in *KRAS*-mutant

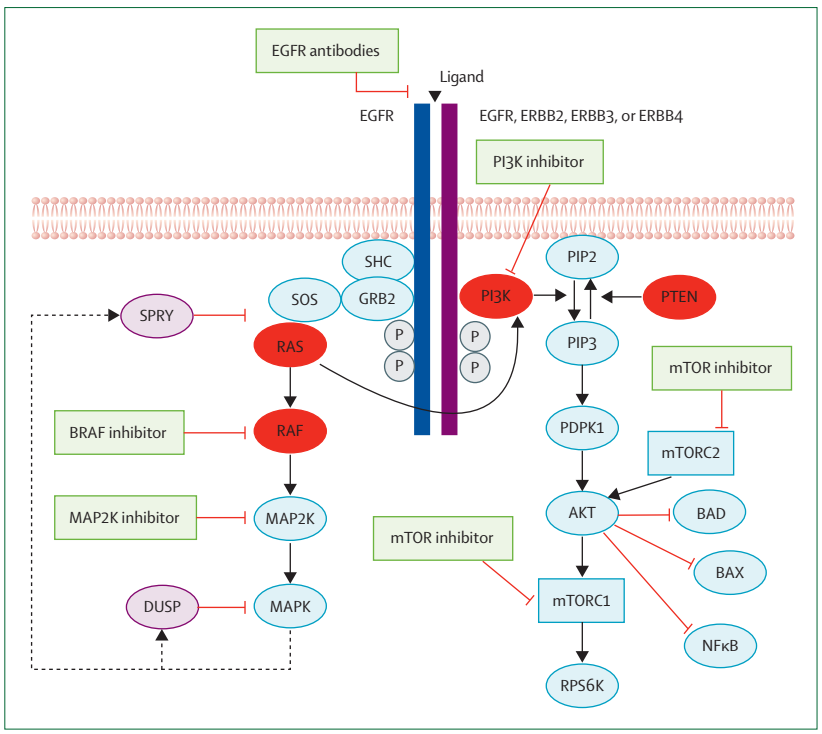

Figure: **EGFR-mediated signalling pathways and possible targets for therapy**
Mediators affected by oncogenic alterations are shown in red. Two feedback mechanisms are shown in purple. EGFR=epidermal growth-factor receptor. PI3K=phosphatidylinositol 3-kinase. PTEN=phosphatase and tensin homologue. PIP2=phosphatidylinositol 4,5-bisphosphate. PIP3=phosphatidylinositol (3,4,5)-trisphosphate. PDK1=3-phosphoinositide dependent protein kinase 1. SPRY=sprouty homologue. mTOR=mammalian target of rapamycin. DUSP=dual specificity phosphatase. mTORC2=mTOR complex 2. mTORC1=mTOR complex 1. SOS=son of sevenless. SHC=Src homology 2 domain containing transforming protein. GRB2=growth factor receptor-bound protein 2.

populations.[26] Patients with *KRAS*-mutant colorectal cancer with high DUSP4 mRNA expression had significantly longer progression-free and overall survival after cetuximab treatment than those with low DUSP4 expression.[26]

B-RAF

BRAF, a member of the *RAF* gene family (*BRAF*, *ARAF1*, and *RAF1*), encodes a serine-threonine protein kinase that is a downstream effector of activated KRAS. Roughly 15% of colorectal cancers harbour *BRAF* mutations,[13,27] although this frequency is heavily dependent on the patient population studied, since *BRAF* mutations confer poor prognosis[14,28] and the number of patients with *BRAF*-mutant tumours declines in later lines of therapy.[4,12,14,28] The most frequently reported *BRAF* mutation in tumours (>95%) is the V600E mutation within the kinase activation domain of the B-RAF protein. The signalling changes resulting from a V600E mutation are unclear. In a simple model, there is an increase in MAPK1/3 activation, as seen for mutant *KRAS*, since B-RAF acts downstream of KRAS to activate MAP2K (figure). However, the V600E mutation could have additional functions, since *KRAS* and *BRAF* mutations

	Comments
***KRAS* mutation (already implemented in clinical practice)**	
Analysis from five single-group studies and five randomised clinical trials in first-line and chemotherapy-refractory mCRC[11]	*KRAS* codon 12 and 13 mutations were significantly associated with non-response and shorter median PFS and OS after treatment with cetuximab (with or without chemotherapy) or panitumumab, regardless of line of therapy
European consortium: 649 patients with chemotherapy-refractory mCRC given cetuximab+chemotherapy[12]	*KRAS* mutations (including codon 61, excluding codon 146) were significantly associated with non-response and shorter median PFS and OS
***BRAF* mutation**	
79 patients with *KRAS*-wild-type chemotherapy-refractory mCRC given panitumumab-based or cetuximab-based treatment[13]	*BRAF* mutations were significantly associated with non-response and shorter median PFS and OS
116 patients with *KRAS*-wild-type chemotherapy-refractory mCRC given cetuximab-based treatment[4]	*BRAF* mutations were significantly associated with shorter median PFS and OS and there was an association with non-response
European consortium: 370 patients with *KRAS*-wild-type chemotherapy-refractory mCRC given cetuximab+chemotherapy[12]	*BRAF* mutations were significantly associated with non-response and shorter median PFS and OS
625 patients with *KRAS*-wild-type mCRC given first-line cetuximab+FOLFIRI or FOLFIRI alone (CRYSTAL randomised trial)[14]	*BRAF* mutants benefit slightly from the addition of cetuximab to FOLFIRI in terms of objective response, PFS, and OS
***PIK3CA* mutation**	
77 patients with *KRAS*-wild-type chemotherapy-refractory or chemotherapy-naive mCRC given panitumumab-based or cetuximab-based treatment[15]	*PIK3CA* mutations were significantly associated with non-response and a shorter median PFS but not OS
122 patients with *KRAS*-wild-type chemotherapy-refractory mCRC given cetuximab-based treatment[16]	*PIK3CA* mutations were not associated with non-response
European consortium: 370 patients with *KRAS*-wild-type chemotherapy-refractory mCRC given cetuximab+chemotherapy[12]	*PIK3CA* exon 9 mutations were associated with *KRAS* mutations. Only *PIK3CA* exon 20 mutations were significantly associated with non-response and a shorter median PFS and OS
PTEN loss of expression	
27 patients with chemotherapy-refractory or chemotherapy-naive mCRC given cetuximab+chemotherapy[17]	Loss of PTEN expression was associated with non-response
85 primary tumour samples and 55 metastatic tissue samples from patients with chemotherapy-refractory mCRC given cetuximab+chemotherapy[18]	Loss of PTEN expression in the metastatic tissue (but not in the primary tumour) was associated with non-response and a shorter median PFS
110 patients with chemotherapy-refractory or chemotherapy-naive mCRC given panitumumab-based or cetuximab-based treatment[15]	Loss of PTEN expression was associated with non-response and a shorter median OS
111 patients with KRAS-wild-type chemotherapy-refractory mCRC given cetuximab-based treatment[4]	Loss of PTEN expression was associated with a shorter median OS, but no association was found between PTEN expression and response or PFS

mCRC=metastatic colorectal cancer. PFS=progression-free survival. OS=overall survival. FOLFIRI=folinic acid, fluorouracil, and irinotecan. FOLFOX=folinic acid, fluorouracil, and oxaliplatin. PTEN=phosphatase and tensin homologue.

Table 1: **Oncogenic alterations in downstream effectors of EGFR-signalling and outcome after treatment with antibodies for EGFR**

are mutually exclusive in colorectal cancer,[12] suggesting that they occur in different tumour types and might have different outcomes. The histology and clinical characteristics of *BRAF*-mutant tumours are different from *KRAS*-mutant tumours,[28] which also suggests specificity of the mutation for tumour subtypes. Moreover, *BRAF* mutations are associated with the CpG island methylator phenotype (CIMP) and microsatellite instability, whereas *KRAS* mutations are more common in CIMP-low and microsatellite-stable tumours.[13,28] V600E-mutant *BRAF* has a major negative prognostic effect in colorectal cancer, whereas mutant *KRAS* does not, so other effects of the *BRAF* V600E mutation need to be explored.

In most studies, no response was seen to cetuximab or panitumumab in patients with *BRAF*-mutant mCRC in the chemotherapy-refractory setting.[4,13,29] In a study,[12] two of 24 patients (8·3%) with a chemotherapy-refractory *BRAF*-mutant tumour (of which one was a V600E mutant) responded to cetuximab plus chemotherapy. Taken together, the available data strongly suggest that the *BRAF* V600E mutation confers resistance to EGFR monoclonal antibodies in patients with chemotherapy-refractory *KRAS*-wild-type mCRC. *BRAF* mutations were associated with poor prognosis in patients with *KRAS*-wild-type tumours receiving either cetuximab with folinic acid, fluorouracil, and irinotecan (FOLFIRI) or FOLFIRI alone in the CRYSTAL study.[14] The researchers suggested that *BRAF* mutants gained additional benefit from cetuximab in combination with FOLFIRI, but the sample size was too small to draw conclusions. An absolute benefit would seem unlikely since very few patients with chemotherapy-refractory *BRAF*-mutant tumours benefit from cetuximab[12,15] or panitumumab.[29] It is possible that the addition of a biological drug in the first-line setting could modify the particularly unfavourable prognosis of *BRAF*-mutant patients given chemotherapy. Until the effects of mutant *BRAF* on MAPK or other pathways are known, it is unclear whether patients with *BRAF*-mutant colorectal cancer will benefit from therapies targeting RTK pathways.

PI3K

The PI3Ks are a family of lipid kinases[30] grouped into three classes with different structure and substrate preferences. Activation of class IA PI3Ks is initiated when a growth factor binds to its cognate RTK, which includes members of the ERBB-family, platelet-derived growth-factor receptor (PDGFR), and the insulin and insulin-like growth-factor-1 receptors (IGF1R). Class IA molecules are heterodimers comprising regulatory (p85) and catalytic (p110) subunits. The p110α isoform (encoded by *PIK3CA*) is mutated in around 15–18% of colorectal cancers.[31,32] More than 80% of *PIK3CA* mutations in colorectal cancers occur in exon 9 (60–65%) or exon 20 (20–25%).[12] Mutations in *PIK3CA* can co-occur with *KRAS* or *BRAF* mutations.[12,33] The gain of function induced by exon-9 (helical-domain) mutations is independent of binding to the p85 regulatory subunit, but requires interaction with RAS-GTP. By contrast, exon-20 (kinase-domain) mutations are active in the absence of RAS-GTP binding but are highly dependent on the interaction with p85.[34] It has been speculated that the contrasting roles of p85 and RAS-GTP in helical-domain and kinase-domain mutant *PIK3CA* reflect two distinct states of mutated p110α.[34]

A European consortium recently suggested that only *PIK3CA* exon-20 mutations are associated with a lack of cetuximab activity in *KRAS*-wild-type tumours.[12] By contrast, exon-9 *PIK3CA* mutations are associated with *KRAS* mutations and do not have an independent effect on cetuximab efficacy.[12] However, because of the low frequency of *PIK3CA* exon-20 mutations, these data should be regarded as hypothesis-generating and require confirmation, as well as further biological studies such as gene-expression profiling. The apparent difference between exon-9 and exon-20 mutations could explain the conflicting data regarding *PIK3CA* mutations. Sartore-Bianchi and colleagues[15] reported that *PIK3CA* mutations are associated with lack of response to EGFR monoclonal antibodies, but their cohort contained more exon-20 mutations and less exon-9 mutations than the series by Prenen and colleagues,[16] in which no correlation was noted between *PIK3CA* mutation status and response to cetuximab.

PIK3CA mutations as a whole were associated with shorter cancer-specific survival in a series of *KRAS*-wild-type stage I–III colorectal cancer,[35] but exon-9 and exon-20 mutations were not studied separately. More studies are needed to establish the prognostic role of *PIK3CA* exon-9 and exon-20 mutations. By contrast with the weak global effect of mutant *PIK3CA* on EGFR inhibition in colorectal cancer, strong effects were seen for trastuzumab in ERBB2 (also called HER2)-positive breast cancer.[36] ERBB2 and ERBB3 signalling strongly activate PI3K, whereas EGFR mainly activates MAPK1/3, which could explain the different effects of *PIK3CA* mutations on different RTK inhibitors. Tissue specificity might also have a role, and it will be interesting to see if the findings regarding the effects of mutations in *PIK3CA* exons 9 and 20 hold true in breast cancer. Since PI3K is also an important mediator in the IGF1R pathway, it is possible that *PIK3CA* mutation status will be an important factor in predicting outcome to IGF1R inhibitors.

PTEN

PI3K-initiated signalling is inhibited by phosphatase and tensin homologue (PTEN). PTEN activity can be lost through various mechanisms, including mutations in *PTEN* (5% on average, with higher frequency in tumours with high microsatellite instability), allelic losses at chromosome 10q23 (23%), or hypermethylation of the *PTEN* promoter region (19·9% in colorectal cancer with high microsatellite instability *vs* 2·2% in low microsatellite instability).[37] These data of PTEN alterations in colorectal cancer are tentative because they come from a small series.

In ERBB2-overexpressing breast cancer, trastuzumab needs intact PTEN for a therapeutic response, and PTEN loss predicts trastuzumab resistance.[38] However, the role of PTEN loss in colorectal cancer is unclear. It has been suggested that loss of PTEN expression, as measured by immunohistochemistry, is associated with lack of benefit from cetuximab in mCRC.[4,17,18,33] Loss of PTEN has been found to co-occur with *KRAS*,[4,33] *BRAF*,[4,33] and *PIK3CA* mutations,[33] and *EGFR* polysomy.[4] However, the recorded frequency of loss of PTEN expression varies from 19% to 36%, with some studies reporting an effect on response rate and survival, whereas others found an effect only on progression-free or overall survival. Moreover, data on the loss of PTEN expression are not concordant in primary and metastatic tissues.[18] Since there is currently no standardised method for PTEN expression analysis by immunohistochemistry, PTEN expression data cannot be reliably used for outcome analyses.

Acquired resistance to EGFR antibodies

Patients with mCRC that initially respond to EGFR monoclonal antibodies eventually become resistant to these drugs. The duration of response depends on the time that it takes cancer cells to develop resistance mechanisms. Resistance might occur through the selection of clones that are resistant at the start of treatment, or the development of acquired resistance in cancer cells that are initially sensitive to treatment. Various molecular mechanisms are likely to have a role in acquired resistance to EGFR monoclonal antibodies (panel). Although few data are available for mCRC, important information can be derived from studies of other tumour types.

The T790M mutation of the *EGFR* tyrosine-kinase domain causes resistance of non-small-cell lung cancer (NSCLC) cells to EGFR tyrosine-kinase inhibitors (TKIs).[39] However, *EGFR* mutations are rarely found in colorectal cancer[40] and, when they do occur, do not affect sensitivity

***Panel:* Potential mechanisms of acquired resistance to anti-EGFR drugs**

EGFR changes in cancer cells

Gene mutations

Downregulation of the receptor

Altered subcellular localisation (nuclear localisation)

EGFR-dependent mechanisms

Activation of downstream signalling pathways through EGFR-dependent mechanisms

- Other cell membrane growth factor receptors (IGF1R; MET)
- PI3K/AKT pathway
- RAS/MAP2K/MAPK pathway
- Proangiogenic growth factors (VEGF) production
- Expression of VEGF in cancer cells

Epithelial to mesenchymal transition

EGFR=epidermal growth factor receptor. VEGF=vascular endothelial growth factor. PI3K=phosphatidylinositol 3-kinase.

to EGFR monoclonal antibodies[5] or TKIs.[41] A reduction or loss of EGFR expression or altered subcellular localisation of the receptor might lead to resistance to EGFR monoclonal antibodies. NSCLC cells with acquired resistance to cetuximab show increased nuclear localisation of EGFR, mediated by SRC family kinases.[42] The relative contribution of this mechanism to resistance to anti-EGFR drugs in colorectal cancer requires investigation.

The main mechanism through which cancer cells could become resistant to anti-EGFR drugs is activation of intracellular signalling pathways. For example, acquired resistance to EGFR-targeted drugs mediated by the activation of either the RAS/MAP2K/MAPK1/3 or the PI3K/AKT pathways has been shown in head and neck, prostate, and breast-cancer cells.[43,44] In these settings, activation of the pathways seems not to be caused by mutational events, which are more likely to occur in the early phases of cancer development and de-novo drug resistance, but are driven by increased growth-factor signalling through receptors other than EGFR. Acquired resistance to EGFR-targeted drugs in breast-cancer cells involves activation of an IGF1R/PI3K/AKT pathway that is induced by increased synthesis of IGF2.[45] Compared with normal colonic mucosa, overexpression of IGF1R occurs in more than 90% of colon primary adenocarcinomas.[46] Activation of IGF1R in colorectal-cancer cells leads to increased activation of EGFR, which is probably mediated by IGF1R-induced release of transforming growth factor α (TGFα).[47] A synergistic antitumour effect of combinations of anti-EGFR and anti-IGF1R small molecules inhibitors has also been shown in colorectal-cancer cell lines.[46] More importantly, over-expression of IGF1 has been correlated with resistance to cetuximab in *KRAS*-wild-type colorectal cancer.[47] These findings are from retrospective analyses of small groups of patients and require confirmation in a larger cohort.

In NSCLC, amplification of *MET* leads to acquired resistance in about 25% of patients given EGFR TKIs.[48] More recently, MET ligand (hepatocyte growth factor [HGF])-mediated resistance to EGFR TKIs through activation of PI3K, via MET phosphorylation, has been shown.[49] Compared with normal mucosa, increased expression of MET mRNA or protein (or both) has been shown in 50–80% of primary colorectal cancers, and has been correlated with depth of invasion, lymph-node metastases, and worse clinical outcome.[50,51] Activating *MET* mutations have not been reported in colorectal cancer; however, coexpression of *MET and HGF* has been shown, and patients with high expression of both ligand and receptor have a worse prognosis.[52] Since *MET* overexpression is found in most colorectal cancers, it is likely to occur in both *KRAS*-wild-type and *KRAS*-mutant tumours, and cooperation between MET signalling and KRAS signalling in promoting the growth of colorectal-cancer cells has been shown.[53] Expression of *MET* is an early event in colon carcinogenesis—it is found in dysplastic aberrant crypt foci, the earliest neoplastic lesion of colorectal cancer—implying that its expression might be regulated by genes involved in initiating colorectal cancer tumorigenesis.[54] Indeed, *MET*-expression is regulated by WNT/CTNNB1 (β-catenin) signalling.[54] Finally, blocking MET signalling can substantially reduce the growth of colorectal-cancer cells in vivo, suggesting that this receptor might be an important target for therapeutic intervention.[55]

ERBB3 expression has been described in 35–80% of primary or metastatic colorectal cancers[56] and neuregulin 1 (NRG1)-mediated activation of ERBB3 has been shown to activate anti-apoptotic signals in colorectal-cancer cells through AKT.[57] An in-vitro model was the basis of a recent hypothesis that heregulin-ERBB3-ERBB2 loop is involved in acquired resistance to cetuximab.[58] A correlation was also found between an increase in heregulin after cetuximab administration and resistance to the drug in a small cohort of patients with mCRC (n=38).[58]

Human colorectal-cancer cells selected in vivo for their resistance to cetuximab (GEO-CR cells) showed increased *FLT1* (also known as vascular endothelial growth factor receptor [VEGFR] 1) expression and activation compared with parental cetuximab-sensitive cells.[59] Blockade of VEGFR1 with vandetanib, an inhibitor of EGFR, FLT1, and KDR (also known as VEGFR2), or specific silencing of *FLT1* through siRNA, significantly reduced the growth and migration of cells with acquired resistance to anti-EGFR drugs, suggesting that VEGFRs might mediate resistance of cancer cells to anti-EGFR drugs.

Cancer cells can undergo an epithelial-to-mesenchymal transition (EMT), an event that contributes to tumour progression and intratumoral heterogeneity. Several lines of evidence suggest that EGFR signalling, in addition to the IGF1R and the TGFβ pathways, could trigger EMT.[60] However, once EMT is established, signalling associated with EGFR activation is attenuated and does not have a

major role in controlling proliferation and survival of mesenchymal-like cancer cells. In NSCLC cells with mesenchymal-like features, tyrosine phosphorylation of ERBB receptors and expression of EGF-like growth factors is decreased, and aberrant expression of PDGFR and fibroblast growth-factor receptor (FGFR) that activate both MAP2K/MAPK1/3 and PI3K/AKT signalling occurs.[61] In colorectal-cancer cells, EMT has also been associated with increased VEGF secretion and increased *FLT1* expression, which sustain the survival of the tumour cells through an autocrine pathway.[62] In agreement with these findings, cell lines from NSCLC, pancreatic, and colorectal cancer that express mesenchymal-like markers are relatively resistant to the EGFR TKI erlotinib, compared with cell lines expressing epithelial markers.[63] In the TRIBUTE trial,[63] among those given erlotinib, a small subgroup of patients with tumours staining strongly for E-cadherin (epithelial marker) had a longer time to progression. However, the importance of this finding is limited by the small size of the subgroup analysed for E-cadherin expression (8% of enrolled patients). Finally, we have previously noted that the blockade of teratocarcinoma-derived growth factor 1 (TDGF1) (also known as CRIPTO), which is associated with EMT, inhibits colorectal-cancer cell growth and has a synergistic antitumour effect when combined with inhibition of EGFR signalling.[64] Therefore, it is likely that EMT might have an important role in resistance to anti-EGFR drugs by activating alternative signalling pathways.

Targeted therapies

To overcome resistance to EGFR monoclonal antibodies in patients with mCRC, novel targeted therapies are being extensively tested in the preclinical setting and are rapidly entering clinical trials. However, prediction of who will benefit from these novel drugs will be more difficult than first anticipated.

MAP2K inhibitors

MAP2K is a downstream effector of B-RAF. The two MAP2K isoforms, MAP2K1 and MAP2K2, share two consensus kinase motifs, one involved in phosphorylation of serine-threonine residues and another in phosphorylation of tyrosine residues. The only known substrates for both MAP2K isoforms are MAPK1 (also called ERK2) and MAPK3 (also called ERK1).[65] Most of the MAP2K inhibitors target both MAP2K1 and MAP2K2. MAP2K inhibitors show substantial preclinical activity in tumour models harbouring V600E *BRAF* mutations. Solit and colleagues[66] showed that mutant *BRAF* is predictive of sensitivity to MAP2K inhibition in vitro. This inhibition caused a decline in cyclin-D1 protein expression, induction of G1 arrest, and apoptosis. In *BRAF*-wild-type cells, even when phosphorylated-MAPK inhibition was completely achieved, no effect was seen on cyclin-D1 expression and tumour growth. Phosphorylated MAPK might not be a good measure of MAPK activity.[25] After treatment of V600E *BRAF*-mutant tumour cells with a selective MAP2K inhibitor, a set of 52 genes was identified whose expression changed rapidly, which included transcription factors associated with MAPK-dependent transformation as well as feedback regulators of MAPK signalling, such as DUSP6.[21] By contrast, such a set of genes could not be identified after RTK activation in tumour cell lines without a *BRAF* mutation. Therefore, DUSP6 expression or phosphorylated MAP2K could be proposed as biomarkers of the RAF/MAP2K/MAPK pathway output and not phosphorylated MAPK.

Additionally, *RAS*-mutant tumour cells were not as sensitive to MAP2K inhibition as *BRAF*-mutant cells.[66] However, it is well known that the KRAS protein has several downstream effector pathways that are not blocked by inhibiting MAP2K. In the context of an *APC*-mutant mouse model of colorectal cancer, RAF, but not MAP2K, seemed to act as a crucial mediator of KRAS signalling.[67] A phase 2 study of the MAP2K inhibitor, AZD6244, in cancers harbouring *BRAF* mutations (identified by prospective genotypic analysis) is now recruiting.

A range of MAP2K inhibitors have been, or are being studied, in phase 1 and 2 trials. As yet, none have reached approval from th US Food and Drug Administration or European Medicines Agency. Development of several compounds was stopped because of ocular toxicity or very low response rates.[65] It is crucial to identify cancers that are likely to respond to MAP2K inhibitors. *BRAF*-mutant tumours might be good candidates, but in the case of *KRAS*-mutant tumours, where KRAS signalling has several downstream effectors, a combination of targeted drugs seems appropriate. Evidence suggests that not all *KRAS* mutations are similar—eg, codon-146 mutations do not have an effect on cetuximab efficacy.[12] Moreover, heterogeneity in KRAS signalling is suggested by the finding that patients with *KRAS*-mutant colorectal cancer with high DUSP4 mRNA expression have a significantly longer progression-free and overall survival after cetuximab than patients with low expression.[26]

B-RAF inhibitors

Sorafenib is one of the best-studied B-RAF inhibitors. It is not a specific B-RAF inhibitor, but a multitargeted kinase inhibitor (VEGFR1, VEGFR2, PDGFR-β, Raf-1, SCFR [stem-cell growth factor receptor], and both wild-type and mutant B-RAF). The antitumour effects of sorafenib are, therefore, not uniquely due to B-RAF inhibition.

By contrast, PLX4720 is a potent and selective inhibitor of V600E B-RAF protein.[68] Consistent with high selectivity, MAPK phosphorylation was potently inhibited by PLX4720 in V600E *BRAF*-bearing tumour cell lines, but not in cells lacking oncogenic *BRAF*. Cell-cycle arrest and apoptosis were also exclusively induced in V600E *BRAF*-bearing cells. In V600E *BRAF*-dependent tumour xenograft models, PLX4720 caused substantial delays in tumour growth, including tumour regressions, without evidence of toxicity.[68]

PLX4032 (RG7204) is a V600E B-RAF inhibitor with pronounced activity in patients with *BRAF*-mutant melanoma.[69] However, the clinical activity of PLX4032 in previously treated patients with *BRAF*-mutant mCRC was more modest, with only a 5% (one in 19) response rate.[69]

GDC-0879 is also a highly selective and potent RAF small-molecule inhibitor. In GDC-0879-treated mice, both cell-line-derived and patient-derived V600E *BRAF* tumours exhibited stronger and more sustained pharmacodynamic inhibition and improved survival compared with *KRAS*-mutant tumours.[70] The responsiveness of V600E *BRAF* melanoma cells to GDC-0879 could be substantially changed by modulation of PI3K-pathway activity, since PTEN knockdown induced GDC-0879 resistance in *BRAF*-mutant cancer cells.[70] Although B-RAF is an important mediator of KRAS, B-RAF inhibitors do not seem to be very effective in *KRAS*-mutant tumours. Moreover, it was recently shown that GDC-0879 and PLX4720 activate the RAS/MAP2K/MAPK pathway in *KRAS*-mutant tumours in a RAS-dependent manner, thus enhancing tumour growth in xenograft models.[71]

PI3K inhibitors and mTOR inhibitors

Inhibition of PI3K could be another approach for treatment of tumours resistant to EGFR monoclonal antibodies because of abnormal PTEN/PI3K status. The PI3K inhibitors wortmannin and LY294002 cause substantial growth inhibition across a broad spectrum of cancer cell lines when administered as single drugs, particularly in cases of excess PI3K activity.[30] However, these compounds have not progressed to clinical trials because of poor selectivity and high toxicity in animal models.[30] Several new compounds have been developed with the intention of improving pharmacokinetic profiles and target specificity, thus minimising toxicity.[30] Some of these drugs are selective PI3K inhibitors; others are dual PI3K and mammalian target of rapamycin (mTOR) inhibitors. Selective mTOR inhibitors have also been developed. mTOR is a serine and threonine protein kinase that acts downstream of PI3K; it forms complexes in vivo, such as mTORC1, which phosphorylates RPS6K1 and is a downstream effector of AKT, and mTORC2, which phosphorylates AKT on serine 473.[30,72] It should be emphasised that rapamycin analogues of mTOR are active only on mTORC1, whereas inhibitors of the catalytic site are active on both mTORC1 and mTORC2 complexes. Furthermore, dual PI3K and mTOR inhibitors might prevent the activation of AKT, which is noted in some tumours after blockade of mTOR.[73]

Several compounds, including PX-866 (PI3K inhibitor), BEZ235, BGT226, PF04691502 (inhibitors of PI3K and mTOR), GDC-0941 (PI3K inhibitor and weak mTOR inhibitor), SF1126 (inhibitor of PI3K and mTOR), and temsirolimus (rapamycin analogue), are currently being studied in phase 1 and 2 trials.[30] Some of these trials are only recruiting patients with tumours carrying molecular alterations of *PIK3CA* or *PTEN*, or both.

	Possible treatment
KRAS	MAP2Ki+PI3Ki or mTORi, EGFRi+MAP2Ki
BRAF	MAP2Ki, BRAFi
PIK3CA	PI3Ki or mTORi
PTEN	PI3Ki or mTORi, EGFRi
KRAS and *PIK3CA*	MAP2Ki+PI3Ki or mTORi
KRAS and *PTEN*	MAP2Ki+PI3Ki or mTORi
BRAF and *PIK3CA*	MAP2Ki+PI3Ki or mTORi
BRAF and *PTEN*	MAP2Ki+PI3Ki or mTORi, BRAFi+PI3Ki or mTORi
PIK3CA and *PTEN*	PI3Ki or mTORi

i=inhibitor. PI3K=phosphatidylinositol-3 kinase. mTOR=mammalian target of rapamycin. EGFR=epithelial growth-factor receptor. PTEN=phosphatase and tensin homologue. *Specific mutations: *KRAS*, codons 12, 13, 61; *BRAF*, V600E; and *PIK3CA*, exon 20.

Table 2: **Possible treatment options for tumours harbouring specific gene mutations***

Combining targeted drugs

Because of the crosstalk between many of the RTK-signalling pathways, no single gene dependency is to be expected. Moreover, when cancer cells are treated with drugs that block a single molecular target, they are often able to activate alternate pathways as escape mechanisms to overcome the blockade and therefore the effectiveness of these drugs. However, only a small number of trials are studying drug combinations, mainly because most drug companies will not collaborate with other companies. Rational combinations of targeted treatments to circumvent, reverse, or even preclude resistance are therefore necessary for optimum use of molecular targeted therapies in cancer (table 2).

Wee and colleagues[74] reported that *KRAS*-mutant colorectal-cancer cell lines seem completely resistant to MAP2K inhibition when loss of PTEN expression occurs, and partially resistant in the presence of *PIK3CA* mutations. Activation of MAPK signalling after inhibition of mTOR signalling has also been described.[75] Therefore, combination of a MAP2K inhibitor and an mTOR and PI3K inhibitor seems to be a rational approach. Zhang and colleagues[76] combined the mTOR inhibitor rapamycin with the MAP2K inhibitor PD89059 in *KRAS*-mutant colorectal-cancer cell lines. This combination inhibited cell proliferation, caused cell-cycle arrest, and induced apoptosis. Engelman and colleagues[77] engineered a lung-adenocarcinoma mouse model initiated and maintained by expression of mutant *PIK3CA*. Treatment of these tumours with BEZ235 (a pan-PI3K and mTOR inhibitor) led to marked tumour regression. By contrast, mouse lung tumours driven by mutant *KRAS* did not substantially respond to single-drug BEZ235. However, when BEZ235 was combined with a MAP2K inhibitor (AZD6244) there was marked synergy in shrinking *KRAS*-mutant tumours.[77] Mirzoeva and colleagues[78] showed that some breast-cancer cell lines exhibited strong feedback

activation of AKT after MAP2K inhibition. Activation of AKT was dependent on EGFR activation. The combination of MAP2K inhibitors and PI3K inhibitors led to synergistic growth inhibition in these cell lines.[78] This MAP2K-EGFR-PI3K negative-feedback loop requires further investigation in other types of cancer, such as colorectal cancer. It was recently shown that immortalised human-breast epithelial cells carrying alterations in the PI3K pathway were responsive to the rapamycin derivative everolimus, except when *KRAS* mutations occurred concomitantly or were exogenously introduced.[79] Combining MAP2K inhibitors and PI3K and mTOR inhibitors could be a promising strategy in *KRAS*-mutant and *BRAF*-mutant colorectal cancer. Furthermore, this treatment strategy should be investigated in *PIK3CA*-mutant tumours; because of crosstalk between RTK pathways, these tumours might also benefit from the combination of MAP2K inhibitors and PI3K inhibitors, or PI3K inhibitors and EGFR inhibitors.

Di Nicolantonio and colleagues[13] showed that although V600E *BRAF*-mutant colorectal-cancer cell lines were less sensitive to cetuximab than *BRAF*-wild-type cell lines, growth of V600E *BRAF*-mutant cells was substantially inhibited when treated with the combination of cetuximab and sorafenib. This treatment combination is currently undergoing clinical assessment in mCRC in a trial sponsored by the National Cancer Institute (NCT00326495). As mentioned, sorafenib is a multitargeted kinase inhibitor. It would be interesting to evaluate the antitumour activity of combining selective B-RAF inhibitors and selective EGFR inhibitors in the treatment of *KRAS*-mutant and *BRAF*-mutant tumours.

As described previously, the responsiveness of V600E *BRAF* melanoma cells to GDC-0879 could be substantially altered by modulation of PI3K-pathway activity (*PTEN* knockdown induced GDC-0879 resistance in *BRAF*-mutant cells).[70] Thus, the combination of B-RAF inhibitors and PI3K and mTOR inhibitors should be investigated in this setting.

Conclusion

Personalised cancer medicine based on genetic profiling of individual tumours is regarded as the treatment strategy of the future. The discovery of mutant *KRAS* as a predictor of resistance to EGFR monoclonal antibodies has brought this approach into clinical practice in mCRC. However, this seminal finding is only the beginning of a series of novel predictive tools that will affect treatment choices in mCRC. Evidence shows that other molecular alterations, such as *BRAF* and *PIK3CA* (exon 20) mutations, which can co-occur in a single tumour, could preclude response to EGFR monoclonal antibodies. Assessment of the effects of these molecular alterations on the efficacy of new drugs that selectively target proteins involved in EGFR-activated intracellular pathways introduces a new paradigm into clinical oncology. The aim for the near future is personalised anticancer treatments in patients with mCRC, by defining the individual tumour mutation profile of key signalling genes. One good positive predictor would be of much higher interest and would be more feasible to test for in routine clinical practice than the entire range of negative predictors proposed today.[6,14,35] A better understanding of the functional interactions within RTK-activated intracellular pathways is essential to efficiently target the individual tumour and to deliver more effective medical treatments to patients with mCRC.

Search strategy and selection criteria

We searched PubMed and references from relevant articles with the search terms "cetuximab", "mTOR inhibitor", "MEK inhibitor", "BRAF inhibitor", "PI3K inhibitor", "PTEN", "EGFR", "resistance", "panitumumab", "tyrosine kinase inhibitors", and "colorectal cancer". Only articles published in English were included. The search strategy was not limited by date. We also visited the website http://www.clinicaltrials.gov on March 1, 2010, to check for recent clinical trials of the targeted therapies of interest.

Contributors

All authors did the literature search. WDR, VDV, and NN drafted the manuscript. WDR drafted the figure. All authors revised the report.

Conflicts of interest

FC has received honoraria from Merck Serono and Roche. ST has received research grants from Merck Serono. All other authors declared no conflicts of interest.

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