Expert Opinion

KRAS mutations and sensitivity to anti-EGFR monoclonal antibodies in metastatic colorectal carcinoma: an open issue

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Background: Cetuximab and panitumumab, mAbs targeting EGFR, are registered for metastatic colorectal carcinoma (mCRC) patients whose tumors express EGFR as determined by immunohistochemistry. However, this method is not predictive of treatment efficacy. *KRAS*, the human homolog of the Kirsten rat sarcoma-2 virus oncogene, encodes a small G-protein that functions downstream of EGFR-induced signalling. *Objective/Methods*: To examine *KRAS* mutations as predictive factors of response to anti-EGFR mAbs using recently published data. *Results/conclusions*: Several retrospective studies show that efficacy of these mAbs is confined to patients with wild type *KRAS* and genotyping of tumors should be considered before treatment. The absence of *KRAS* mutations does not guarantee an improved likelihood of response to cetuximab and panitumumab. Investigation of other genetic and epigenetic biomarkers will be useful to further refine the responder population. Prospective studies to test the efficacy of combined therapies simultaneously targeting EGFR and the RAS/RAF/MAPK signalling pathways for mCRC are warranted.

Keywords: cetuximab, colorectal carcinoma, epigenetics, KRAS, panitumumab, target therapy

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1. Introduction

Colorectal carcinoma (CRC) is the second largest cause of cancer death both in the United States and in the European Union. In the last decade, by best combining the three active cytotoxics available (fluoropyrimidines, irinotecan and oxaliplatin), median overall survival (OS) of patients with advanced disease has almost doubled from 10 – 12 months to more than 20 months [1].

In recent years research has identified several molecules and processes that play key roles in tumor development and progression and which, therefore, constitute potential targets for novel therapies. The aim of these treatments is to disrupt the signalling process that the cell depends upon for growth and survival. One of the most promising target is the EGFR signalling pathway, which is frequently activated in CRC and has been extensively investigated as a target for cancer therapy. Today, two mAbs directed against EGFR, cetuximab and panitumumab, have been approved by the FDA for the treatment of metastatic CRC (mCRC). The former, a chimeric IgG1 mAb, is used alone and in combination with irinotecan for patients with advanced CRC based on demonstrated improvement in OS [2,3]. Panitumumab, a fully human IgG2 mAb, is generally administered as single agent in subjects with CRC based on an improvement in progression free survival (PFS)

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when compared with placebo [4]. However, only a small proportion (8 – 23%) of patients were observed to achieve an objective response (OR) with cetuximab or panitumumab in these studies. Indeed, cetuximab or panitumumab therapy is costly and might cause side effects.

To optimize benefits and reduce the risks as well as contain costs associated with anti-EGFR therapy, the EGFR has been evaluated as a potential marker of clinical outcomes. In fact, at the time these agents were approved, EGFR testing as determined by immunohistochemistry (IHC) was required. Nevertheless, it is now well established that response to anti-EGFR mAbs does not depend upon the level of expression of EGFR [5]. As a consequence, significant efforts have focused on identifying other potential biomarkers that could predict the response to anti-EGFR antibody therapy [6] in view of the strong interaction between genetic and epigenetic events in tumor evolution. *KRAS*, the human homologous of the Kirsten rat sarcoma-2 virus oncogene, encodes a small G-protein that functions downstream of EGFR-induced cell signalling. *KRAS* mutations are considered early events in the multistep CRC carcinogenesis and are found in approximately 30 – 40% of CRC [7]. The majority (~ 82%) of reported mutations are in codon 12. Mutations at codons 13 and 61 contribute to a lesser degree, accounting for ~ 17% and ~ 1% respectively [8]. Recent clinical data provide growing evidence that *KRAS* mutational status should be used as a molecular marker predictive of anti-EGFR mAb sensitivity in mCRC. Further genetic events such as the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) mutation are actually considered to affect anti-EGFR therapy, whereas very poor data are reported regards the "CpG island methylator phenotype" (CIMP) which seems to be associated to worse prognosis when conventional therapy is used [9,10]. Recently, tumor CIMP status, associated with BRAF mutational status, has been suggested to be useful for stratifying patients with high colon cancer-specific mortality, whereas *KRAS*, still remaining a predictive marker, was unrelated to patients' outcome [11].

2. *KRAS* biology and testing

2.1 Role of *KRAS* in EGFR pathway

RAS protein seems to be a key point in EGFR pathway activation considering its ability in mediating signal transduction by different downstream effectors such as v-raf-1 murine leukemia viral oncogene homolog 1 (RAF-1), Ras-related C3 botulinum toxin substrate (RAC) and phosphatidylinositol 3-kinase (PI3K), with different modes of action [12].

EGFR is a transmembrane receptor activated by external stimuli such as EGF, TGFα, amphiregulin, radiation, drugs, etc., which lead to the immediate autophosphorylation of its cytoplasmatic tyrosine kinase domain. The cytoplasmic EGFR signalling pathway is consisted of four major modules: Phospholipase Cγ (PLCγ)-calcium/calmodulin-dependent protein kinase (CaMK)/protein kinase C (PKC), RAS-RAF-MAPK, PI3K-v-akt murine thymoma viral oncogene homolog (Akt)-glycogen synthase kinase (GSK) and signal transducers and activators of transcription (STATs). Activation of these signalling modules often leads to tumorigenesis, tumour proliferation, metastasis, chemoresistance and radioresistance (**Figure 1**).

PLCγ, via protein kinase C activation acts activating MAPK and c-Jun NH_2-terminal kinase activation which influence proliferation and apoptosis [13]. STAT protein pathways involve phosphotyrosine residues translocating to the nucleus where STAT drives the expression of specific target genes with consequent tumor progression [14]. PI3K is a heterodimeric lipid kinase activated by the interaction with RAS protein and able to activate the protein serine/threonine kinase Akt leading to cell growth, apoptosis resistance, invasion, and migration [12].

The RAS-RAF-MAPK pathway seems to be the most critical for cell proliferation and survival. EGFR activation determines a conformational modification of son of sevenless (Sos) through interaction with growth factor receptor bound protein 2 (Grb2) and Src homology 2 domain containing transforming protein (Shc). Sos is therefore able to induce RAS to activate RAF-1 that, through intermediate steps, phosphorylates the MAPK extracellular signal-regulated kinases 1 and 2. Activated MAPKs are imported into the nucleus where they phosphorylate specific transcription factors involved in cancer growth and progression [15].

Mutations in codons 12 or 13 of *KRAS* gene and valine to glutamic acid substitution (V600E) in *BRAF* gene are mutually exclusive events [16,17] and can constitutively switch on RAS-RAF-MAPK pathway. In particular, among the therapeutic strategies tending to block EGFR, those which act by blocking ligand interactions and downmodulating receptor levels (cetuximab, panitumumab) could be ineffective when KRAS protein is constitutively activated. *KRAS* mutations determine a higher amount of KRAS protein bound to GTP and lead to an oncogenic effect of the protein (**Figure 2**) [8].

In vitro studies provided evidence that the acquisition of a *KRAS* mutation, secondary to EGFR amplification, might be the mechanism by which anti-EGFR mAb-respondent cells become resistant to this therapy. Metastatic colorectal DiFi cell line, with amplification of EGFR and sensitive to anti-EGFR mAb therapy have been transfected with the activated KRAS G12V (Gly12Val) plasmid. These cells showed acquired oncogenic potential and became less sensitive to cetuximab [16].

Interestingly, de Reynies *et al.* analyzed 130 mCRC patients for expression profile and *KRAS* mutations, finding 1220 genes differentially expressed between mutated and non-mutated tumors [18]. A previous expression signature of the response to cetuximab detected 1845 genes differentially expressed in cetuximab responders and non responders [19]. The two lists significantly overlapped showing the same two genes at their top: 5′ nucleotidase, ecto (NT5E) and pleckstrin homology-like domain, family A, member 1 (PHLDA1). These observations lead us to think that the expression of the genes

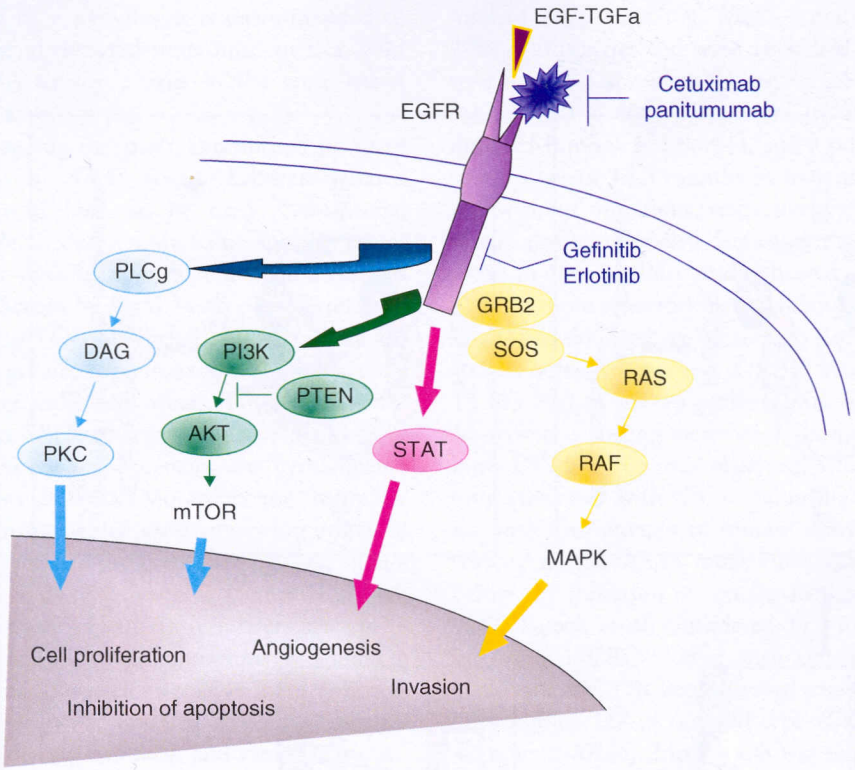

Figure 1. Cellular effects of EGFR activation through signaling cascades. Phospholipase Cγ (PLCγ) via PKC activation acts on proliferation and apoptosis. The signal transducer and activator of transcription (STAT) pathway drives nuclear expression of specific target genes. Phosphoinositol 3 kinase (PI3K) activates v-akt murine thymoma viral oncogene homolog (AKT) leading to cell growth, apoptosis and invasion. Son of sevenless (Sos) can activate *KRAS* involved in RAS-RAF-MAPK pathway, critical for cell proliferation. The mAbs cetuximab and panitumumab, bind to the extracellular domain of EGFR and block signaling cascades.
DAG: Diacylglycerol; GRB2: Growth factor receptor bound protein 2; mTOR: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog.

able to predict anti-EGFR mAb has to take into account *KRAS* alterations.

2.2 *KRAS* mutation testing issue

KRAS determination presents an open question due to the lack of both standardized operating procedures and uniformity of testing method. The large amount of clinical papers based on *KRAS* determination does not take into account that the heterogeneity of results could be biased by many factors including tissue managing and molecular protocols. Formalin-fixed paraffin-embedded (FFPE) archival clinical specimens are inestimable in detecting therapeutic targets even if, in contrast, the suitability of FFPE-derived genetic material results differ with respect to frozen material. In FFPE, in fact, the time and the type of fixation could influence DNA yield and integrity leading to a lack of high molecular weight DNA and the detection of additional genetic changes with respect to the frozen matched counterparts [20]. The reliability of FFPE specimens for recognizing *KRAS* mutations is favored by the small amplicon needed to be analyzed.

Furthermore, DNA extraction protocols are different in different laboratories. Even if commercial kits for nucleic acid isolation are now available, based on solid-phase adsorption (Qiagen, Promega, etc.) or magnetic-bead adsorption (Machery-nagel, Dynal, etc.) or sequential protein and DNA precipitation (Gentra), some laboratories still use home made enzymatic digestion (proteinase k salting-out) or organic solvent extraction (phenol:chloroform extraction). Each protocol permits DNA to be obtained that isdifferent in yield and quality depending on both the amount of starting material and its chemical principles [21].

Because of all these variables in *KRAS* determination, only recently, the European Society of Pathology [22] has been involved in developing guideline procedures and a European Quality Assessment Program for *KRAS* genetic testing [23]. First of all they suggested that patients for whom routine mutation testing has to be performed have to be in stage II or III, then a great importance is accorded to the testing material. The pathologist is responsible for choosing the specimen and for defining the quality and the quantity of the material to be analyzed. In particular, using a hematoxylin–eosin stained section, adequate tumor density (> 70% of invasive carcinoma cells) has to be ensured for *KRAS* mutations detection. One of the main challenges to achieving the right result is the heterogeneity of the testing material. Macrodissection

of the area underlined by a pathologist is recommended to improve the percentage of detected mutations. In fact, wild type and mutant DNA usually coexist in the same lesion and not in equimolar amounts [23].

Another question regards the most appropriate protocol for the determination of *KRAS* status. Laboratory-based methods and commercial kits can be used. Commercial products are often able to detect only some specific mutations and are based on allele-specific PCR [24] and scorpions real time PCR (TheraScreen by DxS) or on peptide-nucleic-acid-based PCR procedure (TIB MolBiol, Trimgen). Laboratory-based methods include gel electrophoresis assays, allele-specific PCR assays, sequencing and many others. The highest sensitivity and specificity are obtained using sequencing protocols with differences between dideoxysequencing and pyrosequencing (personal data not shown). Moreover, the study by Ogino *et al.* [25] demonstrated that pyrosequencing mutation detection limit is 5% on paraffin-embedded tissues, higher than that detected by dideoxysequencing. Recently a high resolution melting analysis (HRMA) has been set up to detect *KRAS*, PIK3CA and *BRAF* mutations providing a reliable and cost-limited approach [26]. Also this method is able to detect at least of 5% of mutated alleles in heterogeneous sample and to identify common and rare mutations.

In this scenario, it is really recommended that all laboratories follow Standard Operating Procedures and accreditation criteria for *KRAS* mutations testing also participating international Quality Control Schemes, when they are organized.

3. Clinical studies in mCRC patients with wild type and mutant *KRAS*

Retrospective analyses of single-arm and randomized studies have investigated the relationship between mutations in *KRAS* and response to anti-EGFR mAbs both in chemorefractory patients and in first-line setting (**Tables 1** and **2**). The goal of these studies is to help clinicians in identifying the subsets of patients who may benefit more from anti-EGFR therapies than others.

3.1 Chemorefractory patients

3.1.1 Single-arm studies

Lievre *et al.* reported for the first time that *KRAS* codon 12 and 13 mutations confer resistance to cetuximab-based treatment and are associated with a poor prognosis when they retrospectively evaluated 30 mCRC [27]. Eleven of the 30 patients (37%) responded to cetuximab. A *KRAS* mutation was found in 13 tumors (43%) and was significantly associated with the absence of response to cetuximab (mutant *KRAS* in 0% of the 11 responder patients versus 68% of the 19 non-responder patients; $p = 0.0003$). The median OS of patients without mutant *KRAS* in their tumor was significantly higher than that of patients with a mutated tumor (16.3 months versus 6.9 months; $p = 0.016$). Subsequently, the same authors presented the results of a larger series including a total of 89 patents [28]. *KRAS* mutations were observed in 27% of this series and were associated with cetuximab-based treatment (0 responders among the 24 patients with mutated tumors versus 40% responders in 65 patients with non-mutated tumors; $p < 0.001$), and a poorer median OS (10.1 months versus 14.3 months in patients bearing tumors with and without mutations, respectively; $p = 0.026$). The multivariate analysis of pooled data concerning these 89 patients and those of the previous study showed that *KRAS* mutational status is more powerful for predicting the resistance to cetuximab than skin toxicity because no patient with mutant *KRAS* had an objective response (OR) to cetuximab compared with 13 (23.2%) of the 56 patients with grade 0 to 1 skin rash. Moreover, a strong correlation between *KRAS* status and both PFS and OS was observed, whereas skin toxicity was only associated with OS. It should be considered that one of the major advantages of mutant *KRAS* compared with skin toxicity is that *KRAS* mutational status can be determined before the initiation of cetuximab treatment.

De Roock *et al.* considered 113 patients with irinotecan refractory mCRC treated with cetuximab with or without irinotecan [29]. OR was observed exclusively in the wild type *KRAS* group (27 of 66 wild type *KRAS* patients versus 0 of 42 mutant *KRAS*). Median OS was significantly better in wild type *KRAS* versus mutant *KRAS* (43.0 versus 27.3 weeks; $p = 0.02$). Interestingly, wild type *KRAS* patients with an initial relative decrease of tumor size > 9.66% at week 6 had a significantly better median OS compared with all other patients (74.9 versus 30.6 weeks; $p = 0.0000025$).

In Di Fiore's trial 59 patients were treated with cetuximab plus chemotherapy [30]. A *KRAS* mutation was detected in 22 out of 59 tumors. Remarkable, no *KRAS* mutations were found in 12 patients with clinical response and time to progression (TTP) was significantly decreased in patients with mutant *KRAS* (3 months versus 5.5 months; $p = 0.015$). Indeed, the author described potential interest regarding the detection of *KRAS* mutations in the circulating tumor cells of mCRC patients [31].

Finocchiaro *et al.* analyzed EGFR, human EGF receptor 2 (HER2), and *KRAS* status in tumor blocks from 85 patients with mCRC treated with cetuximab [32]. Compared with patients with wild type *KRAS*, mutant *KRAS* patients had a significantly lower OR rate (ORR) (6.3% versus 26.5%; $p = 0.02$), shorter median TTP (3.7 months versus 6.3 months; $p = 0.007$), and shorted median OS (8.3 months versus 10.8 months; $p = 0.02$).

Khambata-Ford *et al.* published the results of a pros pective trial enrolling 110 chemorefractory mCRC treated with cetuximab monotherapy [19]. The data showed that the presence of mutant *KRAS* correlated with a lack of response to cetuximab therapy ($p = 0.0003$). Also, patients with tumors that had high gene expression levels of epiregu lin and amphiregulin were more likely to have disease control with cetuximab treatment. The relatively small number of patients exhibiting *KRAS* mutations probably resulted

Table 1. Results of clinical studies in *chemorefractory* mCRC patients according to *KRAS* status.

Study [ref]	KRAS status	Schedule of treatment				
Single-arm studies						
Lievre [27]		***C ± chemotherapy***				
		n patients	**mPFS (wk)**	**p**	**ORR (%)**	**p**
	wt	65	31.4	0.0001	40	< 0.001
	mut	24	10.1		0	
De Roock [29]		***C or C + irinotecan***				
		n patients	**mPFS (wk)**	**p**	**ORR (%)**	**p**
	wt	66	24	0.074	41	0.000001
	mut	42	12		0	
Di Fiore [24]		***C + irinotecan***				
		n patients	**mPFS (mo)**	**p**	**ORR (%)**	**p**
	wt	16	5.5	0.015	28	0.0005
	mut	43	3		0	
Finocchiaro [31]		***C***				
		n patients	**mPFS (mo)**	**p**	**ORR (%)**	**p**
	wt	49	6.3	0.07	26.5	0.02
	mut	32	3.7		6.3	
Khambata-Ford [19]		***C***				
		n patients	**mPFS (dy)**	**p**	**ORR (%)**	**p**
	wt	50	61	ns	10	0.0003
	mut	30	59		0	
Benvenuti [16]		***P or C ± chemotherapy***				
		n patients	**mPFS (dy)**	**p**	**ORR (%)**	**p**
	wt	32	50	0.0443	31	0.073
	mut	16	105		6	
Freeman [32]				P		
		n patients	**mPFS (dy)**	**p**	**ORR (%)**	**p**
	wt	38	16.2	0.002	10.5	0.0028
	mut	24	7.4		0	
Di Nicolantonio [17]		***P or C ± chemotherapy***				
		n patients	**mPFS (mo)**	**p**	**ORR (%)**	**p**
	wt	79	NR	0.027	28	0.011
	mut	34	NR		6	
Randomized studies						
Amado [35]		***P + BSC versus BSC***				
		n patients	**mPFS (wk)**	**p**	**ORR (%)**	**p**
	wt	124 vs 119	12.3 vs 7.3	< 0.0001	17 vs 0	NR
	mut	84 vs 100	7.4 vs 7.3		0 vs 0	
Karapetis [34]		***C + BSC versus BSC***				
		n patients	**mPFS (mo)**	**p**	**ORR (%)**	**p**
	wt	117 vs 114	3.7 vs 1.8	NR	12.8 vs 0	NR
	mut	81 vs 82	1.9 vs 1.8		1.2 vs 0	NR

BSC: Best supportive care; C: Cetuximab; mPFS: Median progression free survival; mut: Mutant; NR: Not reported; ns: Not significant; ORR: Objective response rate; OS: Overall survival; P: Panitumumab; wt: Wild type.

Table 2. Results of clinical studies in mCRC patients in first line setting according to *KRAS* status.

Study [ref]	KRAS status	Schedule of treatment				
Single-arm studies						
Tabernero [38]		***C alone***				
		n patients	**mPFS (wk)**	**p**	**ORR (%)**	**p**
	wt	29	NR		27.6	0.015
	mut	19	NR		0	
		Combination therapy				
		n patients	**mPFS (wk)**	**p**	**ORR (%)**	**p**
	wt	29	9.4	0.047	55.2	ns
	mut	19	5.6		31.6	
Randomized studies						
CRYSTAL [39]		***C + FOLFIRI versus FOLFIRI***				
		n patients	**mPFS (mo)**	**p**	**ORR (%)**	**p**
	wt	172 vs 176	9.9 vs 8.7	0.017	59.3 vs 43.2	0.0025
	mut	105 vs 87	7.6 vs 8.1		36.2 vs 40.2	
OPUS* [41]		***C + FOLFOX versus FOLFOX***				
		n patients	**mPFS (mo)**	**p**	**ORR (%)**	**p**
	wt	61 vs 73	7.7 vs 7.2	0.02	61 vs 37	0.01
	mut	52 vs 47	5.5 vs 8.6	0.02	33 vs 49	
CAIRO2 [44]		***CapOx + B versus CapOx + B + C***				
		n patients	**mPFS (wk)**	**p**	**ORR (%)**	**p**
	wt	152 vs 153	10.7 vs 10.5	ns	NR	
	mut	103 vs 93	12.5 vs 8.6	0.043	NR	

*Phase II randomized study.

B: Bevacizumab; BSC: Best supportive care; C: Cetuximab; CaPox: Capecitabine + oxaliplatin; mut: Mutant; FOLFIRI: Irinotecan with fluorouracil and folinic acid; FOLFOX: Oxaliplatin with fluorouracil and folinic acid; mPFS: Median progression free survival; NR: Not reported; ns: Not significant; ORR: Objective response rate; OS: Overall survival; P: Panitumumab; wt: Wild type.

in insufficient power to detect statistically differences in median PFS.

Freeman *et al.* evaluated the association of *KRAS*, *BRAF*, and PIK3AC gene mutations with tumor resistance to panitumumab alone [33]. From three Phase II panitumumab mCRC studies, 62 of 533 patient samples were available and 24 (38.7%) harboured a *KRAS* mutation. In the wild type *KRAS* group 11% and 53% of patients had a partial response (PR) and a stable disease (SD), respectively. In the mutant *KRAS* group there were no responses.

Benvenuti *et al.* analyzed tumors from 48 patients with mCRC enrolled into clinical trials of cetuximab or panitumumab to assess whether the mutational status of *KRAS* or *BRAF* was associated with the clinical response to anti-EGFR mAbs [16]. In this retrospective analysis the presence of mutant *KRAS* was not significantly linked to OR to therapy, with a trend toward a negative association with response (1 of 11 mutant *KRAS* versus 15 of 37 mutant *KRAS* for responders versus non responders; p = 0.073). Mutant *BRAF* alone was also not significantly associated with OR to therapy. Interestingly, the presence of *KRAS* and/or *BRAF* mutations were negatively associated with PR (p = 0.005).

Di Nicolantonio *et al.* retrospectively analyzed objective tumor response, TTP, OS, and the mutational status of *KRAS* and *BRAF* in 113 tumors from cetuximab- or panitumumab-treated mCRC patients [17]. In this study mutant *KRAS* was present in 30% of patients and was associated with resistance to these mAbs (p = 0.011). Interestingly, the *BRAF* V600E mutation was detected in 11 of 79 patients who had wild type *KRAS*. Indeed, none of the mutant *BRAF* patients responded to treatment, whereas none of the responders carried *BRAF* mutations (p = 0.29), indicating that oncogenic activation of *BRAF* could bypass the EGFR-initiated signalling cascade.

3.1.2 *Randomized studies*

The randomized study CO.17 showed that among 572 patients with mCRC that had not responded to chemotherapy, monotherapy with cetuximab improved OS and PFS better than did best supportive care (BSC) alone [34]. Of the 394 tumors

evaluated for *KRAS* status 41% in the cetuximab group and 42% in the BSC group had a mutation [35]. For patients with wild type *KRAS*, the median PFS was 3.7 months and 1.9 months in the cetuximab and BSC groups, respectively ($p < 0.001$). In the cetuximab group, the ORR among patients wild type *KRAS* was 12.8%, whereas only 1 patient (1.2%) with mutant *KRAS* had a response. None of the patients in the BSC group had an OR. Among patients with wild type *KRAS*, the median OS was 9.5 months in the cetuximab group compared with 4.8 months in the BSC group, with 1-year OS rates of 28.3% and 20.1%, respectively ($p < 0.001$). No differences in terms of OS were observed between patients with mutant and wild type *KRAS* (4.5 months versus 4.6 months). Significantly more patients with wild type *KRAS* than patients with mutant *KRAS* had acne-like rash (95% versus 84%).

Amado *et al.* assessed the predictive role of *KRAS* in 427 evaluable patients with chemotherapy-refractory mCRC and EGFR expression in ≥ 1% of tumor cells (assessed by IHC) receiving panitumumab plus best supportive care (BSC) versus BSC alone [36]. BSC patients could receive panitumumab after disease progression. *KRAS* mutations were found in 43% of patients. The treatment effect on PFS (primary end point) in wild type *KRAS* group was significantly greater ($p < 0.0001$) than those in the mutant group with a median PFS of 12.3 weeks for panitumumab and 7.3 weeks for BSC. Among patients with mutant *KRAS*, however, no benfit was observed from adding panitumumab. RR to panitumumab were 17% and 0%, for wild type and mutant groups, respectively (100% positive predictive value for non-response in the mut group). Median time to response was 7.9 weeks and median duration of response was 19.7 weeks. In the wild type *KRAS* group, 42 (34%) and 14 (12%) patients had SD, respectively. In the mutant *KRAS* group, 10 (12%) patients receiving panitumumab and 8 (8%) BSC patients had SD. Multivariate analyses showed that wild type *KRAS* was significantly associated with OS, but since most patients in the BSC group crossed over, the significance of *KRAS* mutational status was ultimately uncertain. Consistent with longer exposure, more grade III treatment-related toxicities occurred in the wilde type *KRAS* group. No significant differences in toxicity were observed between the wild-type *KRAS* group and the overall population. Importantly, there was demonstrated benefit of panitumumab after cross-over in patients with wild type *KRAS* tumors.

The Phase II randomized study EVEREST (Evaluation of Various Erbitux Regimens by Means of Skin Tumor Biopsies) demonstrated that, in patients with mCRC after failure of irinotecan-based therapy, the efficacy could be improved by escalating the dose of cetuximab (by 50 mg/m^2 every 2 weeks until G2 toxicity, tumor response or dose = 500 mg/m^2) in combination with standard-regimen irinotecan (180 mg/m^2 every 2 weeks) compared with standard dose cetuximab for patients with grade 0/1 skin toxicity [37]. Subsequentely, archived tissue from 77 of 89 randomized patients was analyzed for *KRAS* mutational status with the aims of investigating both the effect of *KRAS* status on treatment outcome and the association between skin toxicity and *KRAS* status [38]. While escalation of the cetuximab dose resulted in a non-significant trend toward higher RR in wild type *KRAS* patients (41.9% versus 30.4% for the standard dose; $p = 0.396$), there was a sharp distinction between outcomes according to *KRAS* status in both treatment arms. The PFS with wild type *KRAS* was 173 days versus 83 days for mutant *KRAS* ($p < 0.0001$). The later group was particularly resistant to response, as dose escalation of cetuximab showed no significant effect and, in fact, the rate of SD was lower for this group. These data suggest that dose escalation does not improve the efficacy in *KRAS*-mutated tumors. Indeed, skin toxicity and *KRAS* status were independent predictors of outcome.

3.2 First line setting

3.2.1 *Single arm studies*

Tabernero *et al.* reported the first series of 48 chemonaive patients initially treated with single-agent cetuximab for 6 weeks and thereafter with cetuximab plus irinotecan with fluorouracil and folinic acid (FOLFIRI) [39].In the cetuximab-alone part of this Phase I/II study, patients with wild type *KRAS* tumors had an ORR of 27.6% compared with 0% for patients with mutant *KRAS* tumors ($p = 0.15$). In the combination part of the study, patients with wild type *KRAS* had an ORR of 55.2% compared with 31.6% for those with mutant *KRAS*. Indeed, median PFS was 9.4 months and 5.6 months in patients with wild type and mutant *KRAS*, respectively ($p = 0.047$).

3.2.2 *Randomized studies*

The CRYSTAL (Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer) study is a Phase III trial with 1198 evaluable mCRC patients with EGFR expression that evidenced, in the Intent to Treat (ITT) population, superiority of the combination FOLFIRI (irinotecan 180 mg/m^2 + 5-fluorouracil (5-FU)/ folinic acid (FA) every 2 weeks) + cetuximab (400 mg/m^2 initial then 250 mg/m^2 weekly) versus FOLFIRI alone in the first line setting in terms of median PFS (8.9 versus 8 months) and RR (47% versus 39%; $p = 0.04$) [40]. Indeed, 1-year survival rate showed an undoubted clinical effect for cetuximab patients (34% versus 23%), mostly for those with liver metastases as the only site of disseminated disease (11.4 versus 9.2 months; $p = 0.02$). Approximately half ($n = 540$) of the ITT patient population was evaluable for *KRAS* analysis [41]. *KRAS* mutations were detected in 35.6% of patients. Median PFS was slightly longer in the *KRAS* population compared with the ITT population (9.2 months with FOLFIRI + cetuximab versus 8.7 months with FOLFIRI). A statistically significant difference in favour of cetuximab was seen in wild type *KRAS* patients for PFS (9.9 versus 8.7 months, $p = 0.0167$) and overall RR (59.3% versus 43.2%, $p = 0.0025$). No significant differences between treatment groups for PFS and ORR were observed in mutant *KRAS* patients. A significant increase

in terms of PFS was documented when cetuximab was added to FOLFIRI in patients wild type *KRAS* (9.9 months versus 7.6 months; p = 0.007) but not mutant *KRAS* (8.1 months versus 8.7 months; p = 0.87). Adverse event profile was similar in wild type and mutant *KRAS* patients. In particular, grade 3 – 4 acne-like rash was as expected in both cetuximab treatment arms (16% versus 17%, respectively).

In the large Phase II randomized OPUS (Oxaliplatin and Cetuximab in First-Line Treatment of Metastatic Colorectal Cancer) study 337 chemonaive patients with mCRC were treated with either FOLFOX (oxaliplatin 85 mg/m^2 + 5-FU/FA every 2 weeks) alone or FOLFOX plus cetuximab (400 mg/m^2 initial then 250mg/m^2 weekly) [42]. Efficacy analysis of this trial in the ITT population failed to show significant improvements in PFS (7.2 months in both arms) and overall RR (35.7% versus 45.6%; p = 0.063), although a significantly higher RR was achieved in subjects with good performance status (36.8% versus 49%; p = 0.032). A retrospective analysis on tissue samples from 233 patients investigated the effect on RR and PFS of patient's *KRAS* status [43]. Mutant *KRAS* was detected in 42% (99/233) of evaluable patients. For wild type *KRAS* patients, the combination had a 61% overall RR, which was significantly improved over FOLFOX alone at 37%, with an odds ratio of 2.54 (p = 0.001). Indeed, in this subset of patients PFS for the combination versus FOLFOX alone was 7.7 months versus 7.2 months (p = 0.016) with a Hazard ratio (HR) of 0.57 and a 43% decrease of the risk of progression. In contrast, in the mutant *KRAS* group, median PFS significantly worsened in patients who received FOLFOX + cetuximab versus FOLFOX alone (5.5 versus 8.6 months; p = 0.019). Overall, the combination FOLFOX + cetuximab demonstrated a survival benefit in patients with wild type *KRAS* compared with patients with mutant *KRAS* (HR: 0.448; p = 0.0009). There was a difference in the pattern of grade 3 – 4 adverse events based on *KRAS* status with an increased incidence of grade 3 – 4 gastrointestinal toxicity in patients with wild type *KRAS* treated with cetuximab compared with those treated with chemotherapy alone or those who had mutant *KRAS* (11.5% versus 5.5% versus 5.8%). Grade 3 – 4 acne-like rash was as expected (14.8% versus 11.5%, respectively).

It should be considered that a potential bias in retrospective analyses of CRYSTAL and OPUS trials is the difference in ITT population versus *KRAS* evaluable population that are made up of 1198 versus 540 (45%) and 337 versus 233 (69%) patients respectively. Moreover, quantitative PCR-based assays used in these studies could identify only seven out of the twelve known *KRAS* gene mutations in exon 2 (codons 12 and 13), and none in exon 3 (codons 59, 61 and 63) [44].

The CAIRO2 (Capecitabine, Irinotecan, and Oxaliplatin in Advanced Colorectal Cancer) study was designed to investigate the effect of adding cetuximab to the combination of capecitabine/oxaliplatin (CapOx) and bevacizumab in 730 untreated patients [45]. In contrast to the expectations, the combined use of CapOx plus bevacizumab and cetuximab had a negative effect on PFS (10.7 months versus 9.8 months, p = 0.019) and left ORR and OS unaffected when compared with CapOx plus bevacizumab alone. *KRAS* mutations were detected in 196 (39%) of 502 patients with evaluable samples. Interestingly, cetuximab did not affect ORR and PFS in wild type *KRAS* patients, while in mutant *KRAS* patients it induced a shorter duration of PFS (8.6 months versus 12.5 months, p = 0.043) and OS (19.2 months versus 24.9 months). These data suggest that, in patients with mutant *KRAS*, the addition of cetuximab to oxaliplatin-based chemotherapy and bevacizumab results in a significant decrease in survival.

4. Expert opinion

EGFR plays an important role in tumorigenesis and tumor progression of CRC. mAbs (cetuximab and panitumumab) targeting EGFR have shown remarkable efficacy in the treatment of patients with mCRC. Neverthless, anti-EGFR drugs are active only in a fraction of patients. Laboratory studies have demonstrated that *KRAS*, which is part of the signalling pathway between EGFR and the cell nucleus, is continually activated when it has specific mutations in codon 12 and 13, even when the EGFR is blocked. Nowadays, several studies provide strong evidence that efficacy of cetuximab and panitumumab is confined to patients with mCRC wild type *KRAS* and that genotyping of tumors should be considered in patients with mCRC before treatment with these drugs. Indeed, EGFR-directed therapy is not only ineffective in mutant *KRAS* mCRC patients, but it could also induce unnecessary toxicity and monetary costs. The European Medicines Agency (EMEA) has already acknowledged these findings by restricting approval of cetuximab and panitumumab to mCRC patients with wild type *KRAS* tumors. Nevertheless, among patients with mCRC wild-type *KRAS* the ORR is limited to 17% (versus 0% in unselected patients) with panitumumab monotherapy [36], 12.8% (versus 1.2% in unselected patients) with cetuximab monotherapy [35], and 59% and 61% (versus 43% and 33% in unselected patients) with cetuximab plus either irinotecan- or oxaliplatin-based chemotherapy [41,43], respectively. These data indicate that the absence of *KRAS* mutations does not guarantee an improved likelihood of response to these drugs. In other words, wild type *KRAS* status is required but not sufficient to confer sensitivity to anti-EGFR mAbs. As a consequence, the investigation of other biomarkers such as EGFR copy number and expression levels of EGFR ligands, phosphatase and tensin homolog (*PTEN*) loss or *BRAF* mutation may be useful to further refine the responder population [17,19,46,47]. However, they are less studied or associated with less consistent data and therefore require prospective analyses. Indeed, it should be considered that clinical studies usually analysed only mutations in codons 12 and 13 of the *KRAS* gene. Edkins *et al.* identified A146 missense substitutions as a new

class of recurrent somatic mutation in the *KRAS* gene in 4% of CRC patients [48]. It is plausible that a further subset of patients are resistant to anti-EGFR mAbs due to A146 mutations. On the other hand, there are few mCRC patients in which the presence of *KRAS* mutations is compatible with a clinical response to anti-EGFR mAbs [16,17,35]. This suggests that in mutant *KRAS* tumors some of the proliferation may still be driven by EGFR signalling and inhibition will result in tumor stabilization. This idea is supported by De Roock's study which showed that a clear decrease in tumor growth rates was observed in mutant *KRAS* patients following initiation of cetuximab therapy. The molecular determinants of response in this subset of patients are presently unknown.

In addition, while *KRAS* mutations occur early in the development of CRC, they may also be subsequently acquired, leading to tumor cell heterogeneity [49]. Considering this genetic heterogeneity, the absence of detectable *KRAS* mutations in the primary tumor could not formally exclude the presence of a mutant *KRAS* in metastases. Several reports demonstrated overall concordance between *KRAS* mutation in the primary tumor and metastasis in CRC [50-52]. In particular, Santini *et al.* in a very large series (99 patients) reported a high concordance between primary and related metastases in terms of *KRAS* mutational status [52]. Further studies evaluating the possible switch of a CRC from wild type to mutant *KRAS* form in patients receiving anti-EGFR mAbs are warranted.

Another criticism of the studies evaluating combinations between anti-EGFR mAbs and chemotherapeutic agents is the possibility that *KRAS* mutation may also influence chemotherapy outcome, irrespective of the administered drug. This hypothesis is the consequence of experimental data on a thymidylate synthase-deficient colon cell line showing that mutant *KRAS* transfection significantly decreased the ability of the cell to undergo apoptosis in response to thymidine deprivation (one of the main mechanism of 5-FU cytotoxicity) [53]. In patients considered in the CRYSTAL and OPUS trials, chemotherapy alone showed the same activity in patients with wild type *KRAS* versus mutant *KRAS* [41,43]. Indeed, a clinical study conducted on 93 mCRC patients receiving 5-FU and leucovorin showed the lack of correlation between the presence of *KRAS* mutations and 5-FU efficacy [54]. These data suggest that any predictive value of *KRAS* status is exclusively linked to the anti-EGFR agents.

Numerous studies examined the treatment-independent effect of *KRAS* status. The Kirsten ras in-colorectal-cancer collaborative group (RASCAL) II study investigating 3439 mCRC patients found that of the 12 possible mutations on codons 12 and 13, only the glycine to valine mutation on codon 12 (8.6%) had a significant effect on survival [55]. However, retrospective data from other large-scale studies has failed to consistently demonstrate a meaningful effect of KRAS mutation on outcome in CRC [56,57].

Lastly, although many diagnostic tools have been developed for *KRAS* mutation analysis, there is an urgent need for validated methods and standardized testing procedures. Recently, guideline recommendations and a European quality assurance program for *KRAS* mutation testing in patients with mCRC have been proposed [23].

In the next 5 – 10 years, we believe that prospective studies will definitely establish the clinical relevance of *KRAS* mutation detection in anti-EGFR mAbs based on chemotherapy. Indeed, future research will test clinical efficacy of combined therapies simultaneously targeting the EGFR and the RAS/RAF/MAPK signalling pathways for mCRC patients in the context of mutational networks affecting the EGFR pathway [58,59].

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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