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REVIEW

Integrated molecular dissection of the epidermal growth factor receptor (EFGR) oncogenic pathway to predict response to EGFR-targeted monoclonal antibodies in metastatic colorectal cancer

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Abstract 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 is widely regarded as a key advance in the field of personalized cancer medicine. Oncologists are now facing emerging issues in the treatment of metastatic colorectal cancer, including: (i) the identification of additional genetic determinants of primary resistance to EGFR-targeted therapy for further improving selection of patients; (ii) the explanation of rare cases of patients carrying *KRAS*-mutated tumors who have been reported to respond to either cetuximab or panitumumab and (iii) the discovery of mechanisms of secondary resistance to anti-EGFR antibody therapies. Here we discuss the potential role of comprehensive dissection of the key oncogenic nodes in the EGFR signaling cascade to predict resistance and sensitivity to EGFR monoclonal antibodies in metastatic colorectal cancer. Current data suggest that, together with *KRAS* mutations, the evaluation of *BRAF* and *PIK3CA*/PTEN alterations could also be useful for selecting patients with reduced chance to benefit from EGFR-targeted therapy. Furthermore, measuring *EGFR* gene copy number also appears relevant to positively identify responders. Up until now, each of these markers has been mainly assessed as a single event, often in retrospective analyses and patients' series. As these molecular alterations display overlapping patterns of occurrence, this adds considerable complexity to the drawing of an algorithm suitable for clinical decision-making. We suggest that in the near future comprehensive molecular analysis of the entire oncogenic pathway triggered by the EGFR should be performed, thus enhancing the prediction ability of individual markers.

Keywords Colorectal cancer · Biomarkers · EGFR · Monoclonal antibodies

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Introduction

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

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metastatic colorectal cancer (mCRC) has been widely regarded as one of the most important recent advances in the field of personalized cancer medicine [1]. Oncologists are now facing a new era in the treatment of mCRC with these drugs, in which unprecedented issues should be addressed. These pertain to: a) the identification of additional genetic determinants of primary resistance to EGFR-targeted therapy for further improving selection of patients, b) the explanation of rare cases of patients carrying *KRAS*-mutated tumors who have been reported to respond to either cetuximab or panitumumab [2–4], and c) the discovery of mechanisms of secondary resistance to anti-EGFR antibody therapies. This article will focus on the first of these issues; indeed, current data suggest that, together with *KRAS* mutations, the evaluations of *BRAF* and *PIK3CA*/PTEN alterations could also be useful for selecting mCRC patients unlikely to respond to EGFR-targeted antibodies. *EGFR* gene copy number detection also appears relevant to positively identify responders. At present, each of these markers has been mainly assessed as a single event, often in retrospective analyses and patients series (Fig. 1), 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.

Beyond *KRAS*: oncogenic activation of other effectors downstream of EGFR that could preclude responsiveness to cetuximab or panitumumab

The occurrence of *KRAS* mutations only accounts for about 30–40% of non-responsive patients and, accordingly, *KRAS* mutations can be considered a highly specific negative biomarker of response (specificity 93%), but they are also poorly sensitive (sensitivity 47%) [3]. The identification of additional genetic determinants of primary resistance to EGFR targeted therapies in mCRC is therefore important to prospectively identify patients who should not receive either cetuximab or panitumumab, thus avoiding their exposure to ineffective and expensive therapy. Recent work has therefore been focused on further molecular dissection of the two main axes that comprise the EGFR signaling cascade. On one side, the KRAS-RAF-MAPK signaling pathway is thought to control cell growth, differentiation and apoptosis. Kirsten (K)RAS belongs to the gene family of oncogenes (KRAS, HRAS and NRAS) encoding guanosine di/tri phosphate (GDP/GTP)-binding proteins that act as self-inactivating intracellular signal transducers. Following Grb2/SOS mediated activation GTP-bound KRAS recruits the serine protein BRAF, thus starting a cytoplasmic phosphorylation cascade leading to the activation of transcription factors [5]. The other axis involves membrane localization of the lipid kinase PIK3CA, which promotes AKT activation ensuing a parallel intracellular propagation of the signal. Importantly, the two axes

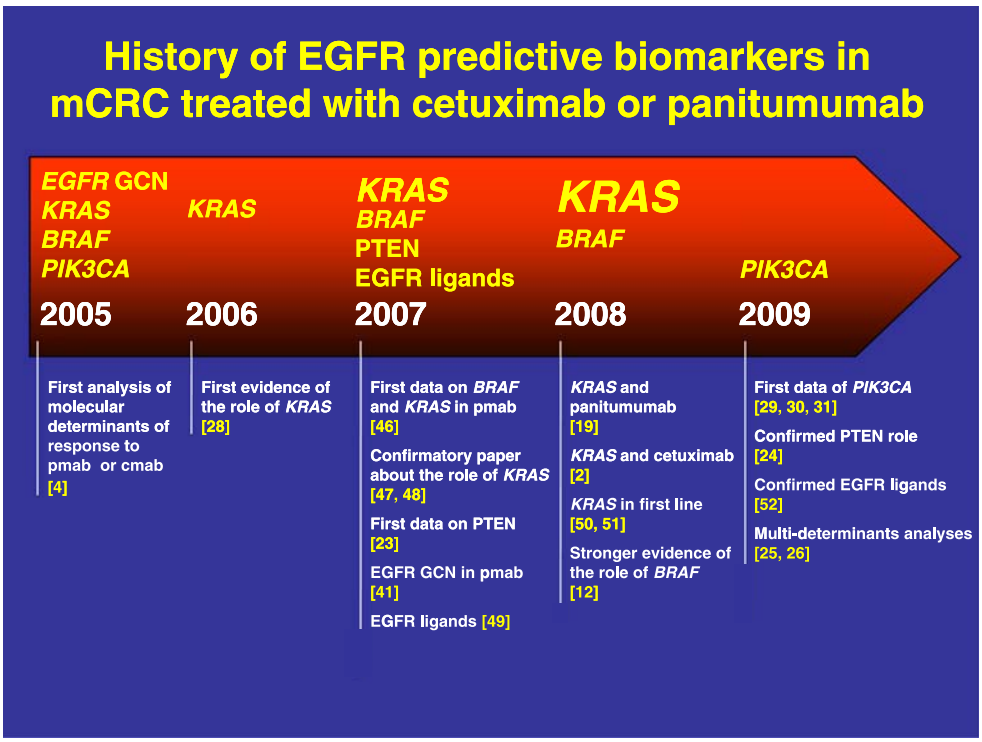

Fig. 1 Timeline of the principal studies assessing the role of molecular determinants of response/resistance to cetuximab or panitumumab in mCRC

(KRAS/BRAF and PIK3CA) are closely related and strictly interconnected, as the p110 subunit of PI3K can also be activated via interaction with RAS proteins. Such close interactions between these axes may provide "escape mechanisms" that allow tumors to circumvent a pathway that has been pharmacologically blocked. Mutations in *KRAS, BRAF* or *PIK3CA* result in continuous activation of the downstream RAS-MAPK or PI3K pathways, regardless of whether the EGFR is activated or pharmacologically blocked. Such activation eventually enhances transcription of various oncogenes, including MYC, CREB, and the gene for nuclear factor κB [6, 7].

BRAF

The $BRAF^{V600E}$ mutation, a thymine to adenine transversion mutation, resulting in the substitution of valine with glutamate, appears in 4–15% of CRC [8–10]. Of note, *KRAS* and *BRAF* mutations are known to be mutually exclusive in CRC [11]. The first analysis assessing the role of the $BRAF^{V600E}$ mutation as a predictive molecular factor to EGFR-targeted therapy was performed in a cohort of 113 tumors from patients who received panitumumab/cetuximab in second or subsequent lines of treatment [12]. 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) (p=0.001) and overall survival (OS) (p<0.001) 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. Furthermore, we showed that the multikinase inhibitor sorafenib may restore sensitivity to EGFR inhibitors in *BRAF*-mutated CRC cell lines [12]. Consequently, combined sorafenib and cetuximab therapy is undergoing clinical evaluation in mCRC in a National Cancer Institute-sponsored trial (NCT00343772; http://clinicaltrials.gov/ct2/show/NCT00343772). Loupakis et al. performed a retrospective analysis among 87 irinotecan refractory patients, treated with anti-EGFR therapy [13]. 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 (median PFS: 2.6 versus 4.4 months in BRAF wild-type, p=0.073) and with significantly shorter OS (median OS: 4.1 versus 13.9 months in BRAF wild-type; p=0.037). Finally, in a wide retrospective cohort of chemo-refractory patients from a European Consortium (n=723), De Roock et al. reported 5% *BRAF* mutations, with a significant association with resistance to cetuximab or panitumumab (objective response = 6% in mutated versus 24% in wild type tumors, p=0.04) [47].

There is now growing evidence that the presence of a $BRAF^{V600E}$ mutation in the primary tumor identifies patients with poorer prognosis, regardless of the treatment regimen (i.e., chemotherapy or chemotherapy combined with EGFR-targeted monoclonal antibodies) and this prognostic value could override its predictive role. In the randomized phase III CRYSTAL study [14] in which patients with mCRC were treated with irinotecan, fluorouracil, and leucovorin (FOLFIRI) or FOLFIRI plus cetuximab as first-line therapy, $BRAF^{V600E}$ mutations were detected in 60 of the 1,000 evaluable patients (6.0%). Patients with tumors containing *BRAF* mutations displayed poorer PFS and OS respect to patients with tumors carrying wild-type *BRAF* in both the FOLFIRI and FOLFIRI plus cetuximab arms, thus supporting the hypothesis that *BRAF* mutational status is a powerful negative prognostic factor, because it is correlated with worse survival that is independent of the type of treatment received. A retrospective analysis of the $BRAF^{V600E}$ mutation status was also performed in 516 tumors from patients treated with first-line cetuximab plus capecitabine, oxalipatin and bevacizumab (CBC) or capecitabine, oxalipatin and bevacizumab (CB) in the CAIRO-2 study [15]. A *BRAF* mutation was detected in 45 tumors (8.7%). In the subgroup of patients treated with CBC the median progression-free interval and OS were 6.6 and 15.2 months for patients with tumors carrying mutant *BRAF* versus 10.4 (p=0.01) and 21.5 (p=0.001) months in those with tumors carrying wild-type *BRAF*. In the subgroup of patients treated with CB the median progression-free interval and OS were 5.9 and 15 months for patients with tumors carrying mutant *BRAF* versus 12.2 (p=0.003) and 24.6 (p=0.002) months in those with tumors carrying wild-type *BRAF*. However, the response rate in the two treatment groups did not differ significantly. The authors concluded that a *BRAF* mutation is a negative prognostic marker in patients with mCRC and that this effect, in contrast to *KRAS* mutations, is not restricted to the outcome of cetuximab treatment [15]. The prognostic value of *BRAF* mutation was highlighted also by Souglakos et al, who determined *KRAS*, *BRAF* and *PIK3CA* mutations in tumors from 168 patients treated with 5-FU-based first-line chemotherapy. Multivariate analysis uncovered *BRAF* mutation as an independent prognostic factor for decreased survival in this setting (HR 4.0, 95% confidence interval [CI] 2.1–7.6). In addition, *BRAF* mutation predicted more rapid disease progression in patients treated with first-line oxaliplatin- (HR 6.4, 95% CI 2.6–15.6), irinotecan- (HR 4.1, 95% CI 1.5–11.3), or oxaliplatin and irinotecan (HR 7.9, 95% CI 1.3–48.2), as well as bevacizumab containing (HR 5.1, 95% CI 2.4–11.1) regimens [16]. In a recently reported analysis, Roth et al. [17] studied resection

specimens (n=1,564) prospectively collected from the PETACC-3 randomized phase III trial assessing the role of irinotecan added to fluorouracil (FU)/leucovorin as adjuvant treatment for stages II and III colon cancer, in order to assess the prognostic role of tumor *KRAS* and *BRAF* status in relation to relapse-free survival and OS. In univariate and multivariate analysis, *KRAS* mutations did not have a major prognostic value regarding relapse-free survival or OS. On the other hand, *BRAF* mutation was not prognostic for RFS, but was for OS, particularly in patients with MSI-low and stable tumors (HR 2.2; 95% CI, 1.4–3.4; p=0.0003). The authors concluded that in stage II–III colon cancer, the *KRAS* mutation status does not have major prognostic value, while *BRAF* is prognostic for OS in MS-L/S tumors.

In conclusion, although a $BRAF^{V600E}$ mutation is being recognized as a prognostic determinant in CRC [14–17], univocal evidence derived from preclinical cellular models [12] and clinical trials of cetuximab or panitumumab in patients with chemotherapy-refractory mCRC indicates that this molecular alteration is also predictive of resistance to EGFR-targeted therapy (Table 1). A negative prognostic role has also been reported for *KRAS* mutations [18], and yet this biomarker is currently used to exclude patients from EGFR-targeted therapy. The large datasets in which the negative predictive value of *KRAS* mutations has been unequivocally established [2, 19] are also ideally suited to assess the role of *BRAF* mutations.

PIK3CA and PTEN

In addition to KRAS and BRAF, the HER family of receptor 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. The role of deregulated PIK3CA/PTEN signaling on the response to targeted therapy has therefore been investigated in breast [20], glioblastoma [21] and also mCRC. The *PIK3CA* mutations occur in approximately 10–18% of CRC patients, principally located in exon 9 and 20 [10, 22], whereas loss of PTEN expression by immunohistochemistry (IHC) is reported in 19–42% [23–26].

In vitro studies in various CRC cell lines have shown that activating *PIK3CA* mutations or loss of PTEN expression appeared to confer resistance to cetuximab. Cell lines that were either *PIK3CA* mutated, or PTEN null, and also had mutations in *RAS* or *BRAF* exhibited the greatest resistance to cetuximab [27]. In the clinical setting, the first three studies analyzing *PIK3CA* mutations [4, 28, 29] reported together nine (10%) tumors bearing PIK3CA mutations and only one responded to EGFR-targeted treatment. In a larger patient series (n=110), we found that *PIK3CA* mutations and PTEN loss in colorectal tumors 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 [30]. 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 OS (p=0.005). This negative association with PFS was also noted in a study by Souglakos et al. [16], where among 92 patients treated using chemotherapy and cetuximab as salvage therapy, *PIK3CA* mutations predicted reduced PFS (2.5 versus 3.9 months, HR 2.1, 95% CI 1.2–3.9). In contrast, Prenen et al. have recently reported in a series of 200 mCRC patients that 23 (12%) carried a *PIK3CA* mutation and five of these (22%) were found in responders [31]. This means that five of 39 responders (13%) and 18 of 160 non-responders (11%) carried a *PIK3CA* mutation, thus not supporting a significant association between *PIK3CA* mutations and lack of reponse to cetuximab (p=0.781). The median PFS and OS did also not differ significantly between *PIK3CA* mutant and wild-type patients. Data from the wide cohort of the European Consortium (n=723) presented at ECCO-ESMO 2009 by Tejpar et al. [47] demonstrated that objective response to EGFR-targeted treatment was lower among patients with tumors carrying *PIK3CA* mutations as compared to wild-type (14% versus 25%, respectively, p=0.03), although this association did not reach statistical significance among *KRAS* wild-type patients (21% versus

Table 1 Available studies of patients with chemorefractory metastatic colorectal cancer and the negative predictive value of the $BRAF^{V600E}$ mutation for response to EGFR-targeted monoclonal antibodies

Author (reference)	No. of patients evaluated with wild-type *KRAS*	No. of patients with $BRAF^{V600E}$ mutations (%)	No. of patients with an objective response/No. of total patients (%)	
			$BRAF^{V600E}$ mutation	Wild-type *BRAF*
Di Nicolantonio [12]	79	11 (14)	0/11 (0)	22/69 (32)
Laurent-Puig [26]	116	5 (4)	0/5 (0)	52/111 (47)
Souglakos [16][a]	92	9 (10)	0/9 (0)	14/83 (17)
Loupakis [13]	87	13 (15)	0/13 (0)	24/74 (32)
De Roock [47]	662[b]	29 (4)	2/29 (7)	156/633 (25)
Overall	1,036	67 (6)	2/67 (3)	268/970 (28)

[a] Subset of patients treated with salvage cetuximab or panitumumab therapy; [b] Not restricted to *KRAS* wild-type

36%, respectively, $p=0.075$). In the same series, *PIK3CA* mutations exerted a detrimental effect on PFS in the whole cohort (18 weeks versus 12.5 weeks, HR 0.74 [0.589–0.928], $p=0.007$), but again this was not statistically significant among *KRAS* wild-type tumor carriers (24 weeks 18 weeks, $p=0.176$). Interestingly, *PIK3CA* mutations showed a borderline association with worse OS both in unselected and *KRAS* wild-type subsets of patients ($p=0.067$).

Ogino et al. studied the prognostic role of *PIK3CA* in specimens from 450 mCRCs that underwent curative surgery (stage I to III) [32]. *PIK3CA* mutations were found in 18% of tumors. Compared with patients with *PIK3CA* wild-type tumors, those with *PIK3CA*-mutated tumors experienced an increase in colon cancer-specific mortality (HR 1.64; 95% CI, 0.95–2.86). Of note, this negative prognostic effect was limited to *KRAS* wild-type tumors, as *PIK3CA* mutation conferred no significant effect on mortality among patients with *KRAS*-mutated tumors.

Frattini et al. [23] reported that none of 11 patients with tumor PTEN loss responded to cetuximab-based treatment, whereas 10 (63%) of 16 patients with intact PTEN protein expression were partial responders. Perrone et al. [29] also noted that none of three patients with PTEN mutations responded to treatment with cetuximab and irinotecan. Razis et al. [33] reported that normal PTEN protein expression was associated with a higher response rate and longer time to progression in patients treated with cetuximab-based therapy, despite a 50% response rate observed in patients who had lost PTEN protein expression. Loupakis et al. performed a retrospective analysis on the status of PTEN in a cohort of 55 metastases from patients with irinotecan refractatory mCRC treated with irinotecan and cetuximab: 12 (36%) of 33 patients with PTEN-positive metastases were responders compared with one (5%) of 22 who had PTEN-negative metastases ($p=0.007$) [24]. The median PFS of patients with PTEN-positive metastases was 4.7 months compared with 3.3 months for those with PTEN negative metastases ($p=0.005$). Patients with PTEN-positive metastases and *KRAS* wild type had longer PFS compared with other patients (5.5 months v 3.8 months; $p=0.001$). In the same study the authors also showed a poor concordance rate between IHC evaluation of PTEN loss between primary tumor and metastases in 45 paired samples (60%), and that PTEN status on primary tumors did not predict response or PFS.

In conclusion, available analyses highlight the role of *PIK3CA* mutations in predicting resistance to cetuximab and panitumumab, although this association is not as strong as the one observed for *KRAS* or *BRAF* mutations, thus not supporting the use of this molecular determinant by itself for clinical decision making. Conflicting results in published works [30, 31] could be, at least partly, explained by the heterogeneity of patients series in terms of the distribution of mutations (i.e., exon 9 versus exon 20), since it has been demonstrated that mutations located in different hotspots exert different biochemical and oncogenic properties and are differently activated by KRAS [34]. As for PTEN, most of the authors agree that its inactivation is a negative predictor of response [23, 24, 30]. This molecular alteration is caused often by epigenetic mechanisms [35], supporting the detection of the intact protein by IHC as a better diagnostic tool than gene sequencing, as it potentially covers more mechanisms of alteration. On the other hand, the lack of standardization of this analysis is likely to affect its clinical application [36]. Moreover, poor concordance rate between expression in primary tumor versus metastatic sites [24] has been reported, introducing further complexity in the molecular diagnostic work-up.

Increased *EGFR* gene dosage as a positive predictive factor of clinical outcome to EGFR-targeted monoclonal antibodies

Contrary to other situations in which the genomic locus corresponding to an oncogene is frankly amplified (*PIK3CA, MET*), thus resulting in largely augmented expression of the corresponding protein product, it seems clear that the increase in *EGFR* gene copy number is often modest (three- to five-fold) and caused by polysomy rather than gene amplification, without a significant increase of the receptor protein [36]. Nevertheless, a biological phenomenon underlying the association between an increase in *EGFR* copy number and positive clinical outcome to EGFR-targeted monoclonal antibodies certainly does exist, as confirmed by different analyses of patients' series (Table 2). This molecular alteration can be detected by fluorescence in situ hybridization (FISH) [37], chromogenic in situ hybridization (CISH) [38] or polymerase chain reaction (PCR), although the latter method seems not to be associated with response to anti-EGFR therapy in published cohorts [39, 40].

A study by our group was the first that demonstrated the association between *EGFR* gene copy number (GCN), determined by FISH analysis, and response to anti-EGFR monoclonal antibodies [4]. We described an 89% response rate in a subgroup of nine patients with colorectal cancer whose tumors had an increased *EGFR* GCN, including a relatively high proportion of responders (9 of 29 patients; 31%) in the analysis. These findings were confirmed from a subsequent study in a retrospective analysis of a subgroup of patients participating in the pivotal phase III trial of panitumumab monotherapy [41]. The mean *EGFR* GCN per nucleus and the percentage of tumor cells with chromosome 7 polysomy (three or more *EGFR* signals

Table 2 Tumor epidermal growth factor receptor gene copy number and outcome of panitumumab- or cetuximab-based treatment in patients with metastatic colorectal cancer

	Author (reference)	Treatment	GCN cutoff; (methodology)	RR (%)	*p*	PFS (months)	*p*	OS (months)	*p*
Unselected population	Sartore-Bianchi [41]	Panitumumab monohterapy	≥2.47	30%	0.0009	8	0.039	15	0.015
			<2.47 (FISH)	0%		3		10	
	Cappuzzo [45]	Cetuximab ± CT	≥2.92	32.6%	0.0001	6.6	0.02	11.3	0.8
			<2.92 (FISH)	2.4%		3.5		8.5	
	Personeni [44]	Cetuximab ± CT	≥2.83	NA	NA	5.5	0.25	10	0.037
			<2.83 (FISH)			4.0		8.3	
	Frattini [23]	Cetuximab ± CT	≥4	22%	0.05	NA	NA	NA	NA
			<4 (FISH)	14%					
	Lievre [28]	Cetuximab ± CT	≥6	27%	0.04	NA	NA	NA	NA
			<6 (CISH)	0%					
	Moroni [4]	Panitumumab or cetuximab ± CT	≥3	89%	<0.001	NA	NA	NA	NA
			<3 (FISH)	5%					
KRAS wild-type population	Scartozzi [42]	Cetuximab + CT	≥2.6	60%	0.002	7.7	0.04	NA	NA
			<2.6 (FISH)	9%		2.9	0.02		
			≥2.12	36%		6.4			
			<2.12 (CISH)	6%	0.03	3.1			
	Laurent-Puig [26]	Cetuximab + CT	≥2.0	71%	0.015	8	ns	16.2	ns
			<2.0 (FISH)	37%		7		11.8	

GCN gene copy number, *RR* response rate, *PFS* progression-free survival, *OS* overall survival, *CT* chemotherapy, *NA* not available, *ns* not statistically significant

per nucleus) were analyzed by FISH and the association between these parameters and clinical outcome was assessed. None of the patients with a mean of 2.47 or less *EGFR* gene copies per nucleus, or fewer than 43% of tumor cells with chromosome 7 polysomy, respectively, achieved objective response, compared with six (30%) of the 20 patients (p=0.001) and six (32%) of the 19 patients (p=0.001) who had values above these thresholds. A mean *EGFR* GCN threshold of less than 2.5 copies per nucleus or fewer than 40% of tumor cells with chromosome 7 polysomy discriminated patients with shorter PFS (p=0.039 and p=0.029, respectively) and OS (p=0.015 and p=0.014, respectively). Moreover, *EGFR* GCN and chromosome 7 polysomy status did not influence progression-free interval in patients receiving only supportive care in this study, suggesting that this parameter is not prognostic in mCRC. The association between *EGFR* GCN increase and response to anti-EGFR therapy, both cetuximab and panitumumab, was reported with different cut-offs by subsequent studies, summarized in Table 2. Most recent studies are now focusing on *EGFR* GCN as an additional determinant of response in *KRAS* wild-type patients; in particular, Scartozzi et al. assessed the role of *EGFR* GCN in 44 irinotecan-refractory *KRAS* wild-type CRC patients treated with irinotecan and cetuximab, using both FISH and CISH techniques [42]. They reported a statistical significant association between GCN and response rate, observing a tumor regression in 9 (60%) and 2 (9%) cases with an increased and low FISH *EGFR* GCN, respectively (p=0.002) and in 10 (36%) and 1 (6%) cases with an increased and low CISH *EGFR* GCN, respectively (p=0.03). Median time to progression was 7.7 and 6.4 months in patients showing increased FISH and CISH *EGFR* GCN, whereas it was 2.9 and 3.1 months in those with low FISH and CISH *EGFR* GCN (p=0.04 and 0.02 respectively) [42]. Laurent-Puig et al. conducted a comprehensive analysis including the evaluation of *EGFR* amplification/polysomy status by FISH and CISH in 96 *KRAS*-wild-type tumors [26]. An *EGFR* FISH-positive phenotype was found in 17 patients (17.7%) who showed a statistically significant higher response rate (71%) respect to patients with normal *EGFR* copy number (37%), p=0.015. A trend toward longer PFS and OS was found in patients with FISH-positive phenotype but without reaching a threshold of significance. The authors used the FISH scoring algorithm developed by Hirsch et al. [43] and reported an accuracy test of 64.9%. Of note, they applied to the same population of patients different FISH scoring algorithms reported in previous papers as useful to discriminate patients with tumor response to cetuximab, in particular an EGFR-to-chromosome probe intensity ratios of 2.47, 2.83 and 2.92 [41, 44, 45], obtaining an accuracy of 57.7%, 63.9%, and 64.9% respectively [26].

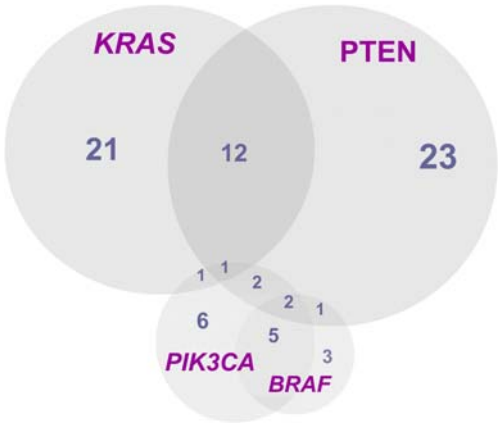

Fig. 2 Representation of the distribution of molecular alterations in individual tumors from a study of 132 patients with metastatic colorectal cancer treated with cetuximab or panitumumab: mutations of *KRAS* and *BRAF* occurred in a mutually exclusive manner, while an overlapping pattern was observed between other alterations [25]

In conclusion, in mCRC the *EGFR* FISH pattern is often not homogeneous, displaying variable degrees of disomy, polysomy or, less, amplification. In this situation, providing a scoring algorithm is difficult, and in order to overcome this problem we proposed to use the percentage of cells showing chromosome 7 polysomy (*EGFR* per nucleus ≥3) or amplification (*EGFR* to CEP7 ≥2) [41] rather than the mean *EGFR* GCN in the examined tumor areas. However, the reproducibility of data remain the largest obstacle for clinical applicability of this molecular determinant and, although eight studies [4, 23, 26, 28, 41, 42, 44, 45] have confirmed its predictive usefulness, methods of tissue processing and *EGFR* scoring systems need to be standardized before using it as a tool for selecting patient for EGFR-targeted therapy.

Multi-determinants analyses to predict clinical outcome to cetuximab or panitumumab

Most recent studies of molecular biomarkers of response to cetuximab or panitumumab are now including comprehensive integrated analysis of different effectors along the pathway triggered by the EGFR, with the aim of enhancing the prediction ability of the markers used individually. In particular, in a recent work [25] we hypothesized that mCRCs could be classified basing on the number of molecular abnormalities detected among known alterations within the EGFR pathway. We retrospectively analyzed objective tumor response, PFS and OS together with the mutational status of *KRAS, BRAF, PIK3CA* and expression of PTEN in 132 tumors from cetuximab or panitumumab treated mCRC patients. Among the 106 non-responsive patients, 74 (70%) had tumors with at least one molecular alteration in the four markers. Of note, while mutations of *KRAS* and *BRAF* confirmed to be mutually exclusive, an overlapping pattern was observed among other alterations, the most frequent overlapping fingerprints being PTEN loss and *KRAS* mutations (co-occurring in 13 patients), and *BRAF* and *PIK3CA* mutations (in 7 patients) (Fig. 2). Among the 96 wild-type *KRAS* patients, loss of PTEN showed a significant association with lack of response ($p<0.001$), while *BRAF* was not significant ($p=0.265$) and *PIK3CA* exerted a borderline effect ($p=0.075$). Survival analyses demonstrated that *BRAF* mutations (HR 3.75, $p=0.015$) and loss of PTEN (HR 0.43, $p=0.009$), but not *PIK3CA* mutations (HR 1.20, $p=0.672$), were significantly associated with decreased OS, whereas none of these alterations was significantly associated with PFS. In light of the occurrence of multiple molecular alterations within the same tumor, we investigated our cohort by separating

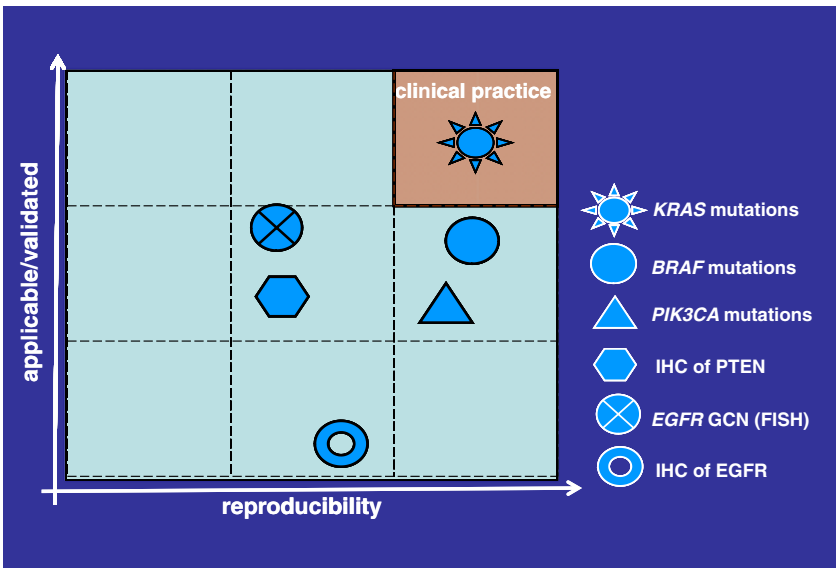

Fig. 3 Diagram representing potential clinical application of principal biomarkers predicting clinical outcome to EGFR-targeted monoclonal antibodies in metastatic colorectal cancer. Each biomarker has been placed basing on its reproducibility and validation from published studies

patients according to the actual number of molecular abnormalities in the same tumor, i.e., none versus 1 versus ≥2 alterations. The probability of response was 51% (22/43) among patients with no alterations, 4% (2/47) among patients with 1 alteration, and 0% (0/24) for patients with ≥2 alterations ($p<0.0001$). Accordingly, PFS and OS were increasingly worse for patients with tumors harboring none, 1, or ≥2 molecular alterations ($p<0.001$). Based on these findings, we proposed a new algorithm that deserves validation in prospective studies for deciding the clinical use of EGFR-targeted monoclonal antibodies. The aim of the multiple molecular testing is indeed to identify mCRCs lacking the three mutations and loss of PTEN ("quadruple negative") as the most sensitive to cetuximab or panitumumab therapy.

Laurent-Puig et al. [26] reported a similar comprehensive analysis of molecular biomarkers, including *EGFR* GCN (see previous section) but not *PIK3CA* evaluation and focusing on *KRAS* wild-type tumors. They retrospectively collected specimens from 173 patients with mCRC treated with a cetuximab-based regimen as ≥ second-line. In patients with KRAS wild-type tumors ($n=116$), *BRAF* mutations (3%) were weakly associated with lack of response ($p=0.063$) but were strongly associated with shorter PFS ($p<0.001$) and shorter OS ($p<0.001$). A high *EGFR* polysomy or an *EGFR* amplification was found in 17.7% of the patients and was associated with response ($p=0.015$). PTEN null expression was found in 19.9% of the patients and was associated with shorter OS ($p=0.013$). In multivariate analysis, *BRAF* mutation and PTEN expression status were associated with OS. The authors concluded that in patients with wild-type *KRAS*, mutations in *BRAF* are associated with lack of response, shorter PFS and OS; *EGFR* amplification is associated with response although clinical decisions based on *EGFR* copy number are not warranted as long as FISH technology and scoring are not standardized; and PTEN protein expression is associated with OS, thus supporting a prognostic role of this determinant.

In conclusion, important advances have been made toward personalized cancer therapy for cetuximab and panitumumab. The molecular dissection of the EGFR pathway proved to be an efficacious strategy for selecting mCRCs to treat with these compounds, as hypothesized by our first research works [4, 46]. Each one of the molecular determinants evaluated in this review indeed demonstrated to affect clinical outcome to EGFR-targeted monoclonal antibodies, although, by itself, only *KRAS* reached the clinical practice (Fig. 3). We feel that the best strategy has been the evaluation of these biomarkers in the context of a multi-determinants analysis including both the KRAS-RAF-MAPK and the PI3K-PTEN-AKT signaling pathways [25, 26], providing predictive algorithms that are ready for validation in prospective trials. Moreover, in a near future, the therapeutic armamentarium for mCRC will be further expanded by introduction of novel targeted agents, and current data from comprehensive integrated analysis of different effectors along the EGFR pathway will support a rational selection among different available options.

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Conflict of interest statement None of the authors declare a conflict of interest.

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