Beyond *KRAS*: perspectives on new potential markers of intrinsic and acquired resistance to epidermal growth factor receptor inhibitors in metastatic colorectal cancer

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***Abstract*:** The monoclonal antibodies cetuximab and panitumumab, directed against the epidermal growth factor receptor (EGFR), are licensed for the treatment of *KRAS* wild-type metastatic colorectal cancer (mCRC). Such 'molecular restriction' derived from *post-hoc* analyses of randomized trials and from other retrospective series all indicate how tumors bearing *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations are resistant to EGFR inhibition. Even if highly sensitive for nonresponse, *KRAS* testing is not very specific. In fact, a limited but still considerable proportion of *KRAS* wild-type patients rapidly progress on treatment with an EGFR inhibitor. New potential molecular determinants of benefit from such treatment are under investigation and may further refine the selection of patients. Pharmacogenomic analyses and translational studies are also ongoing for exploring the field of acquired resistance to anti-EGFRs, since all patients eventually progress. New biological data are awaited for optimizing the use of molecular agents in colorectal cancer and for identifying promising targets that could allow to better understand and, potentially, overcome mechanisms of primary or secondary resistance to EGFR inhibitors.

***Keywords*:** colorectal cancer, epidermal growth factor receptor, cetuximab, panitumumab, predictive factors

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Introduction

In the last few years, the optimization of conventional chemotherapeutic combinations and the introduction of new molecular targeted agents, such as the monoclonal antibodies directed against the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor (VEGF), have considerably improved the prognosis of patients affected by metastatic colorectal cancer (mCRC). At the same time, the wide broadening of therapeutic options has started off a number of intriguing new challenges for oncologists dealing with colorectal cancer (CRC), as, first of all, the choice of the best treatment for the single patient and the single tumor.

Cetuximab and panitumumab are directed against the EGFR, a transmembrane glycoprotein that plays a crucial role in the acquisition of malignant characteristics by neoplastic cells. It takes part in the regulation of cellular growth, proliferation, invasion and migration, by interacting with a variety of intracellular signaling cascades, such as RAS/RAF/MAPKs and PTEN/PI3K/Akt pathways [Herbst *et al.* 2002]. The safety and efficacy of EGFR inhibitors have been proven both as single agents [Van Cutsem *et al.* 2009; Jonker *et al.* 2007; Van Cutsem *et al.* 2007] and in combination with standard chemotherapy regimens in different lines of treatment [Van Cutsem *et al.* 2009; Sobrero *et al.* 2008; Jonker *et al.* 2007; Van Cutsem *et al.* 2007]. *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations occur in about 40% of colon cancers [Andreyev *et al.* 2001] and determine the constitutive activation of RAS protein, which becomes thus independent from EGFR control. *Post-hoc* analyses of

randomized trials [Bokemeyer *et al.* 2009; Van Cutsem *et al.* 2009; Amado *et al.* 2008; Karapetis *et al.* 2008] have demonstrated that anti-EGFR monoclonal antibodies are ineffective in patients bearing *KRAS* codon 12 or 13 mutated tumors, so that the use of these agents is restricted to patients with *KRAS* wild-type disease [Allegra *et al.* 2009].

Consequently, the assessment of *KRAS* mutations has now become the milestone of the selection of patients to be treated with anti-EGFR antibodies.

Bevacizumab, a monoclonal antibody directed against VEGF, is approved in the treatment of mCRC patients in combination with fluoropyrimidine-based chemotherapy [Hurwitz *et al.* 2004], representing a standard first-line therapeutic option in clinical practice. Moving from encouraging preclinical [Ciardiello *et al.* 2000], as well as early clinical studies [Saltz *et al.* 2007], suggesting a benefit from the combination of anti-VEGF and anti-EGFR antibodies, two first-line phase III trials have been recently conducted to assess the efficacy of the double inhibition. Both PACCE [Hecht *et al.* 2009] and CAIRO-2 [Tol *et al.* 2009a] trials reported an unexpected detrimental effect in terms of progression-free survival (PFS) for patients treated with chemotherapy plus bevacizumab and panitumumab or cetuximab, compared with those treated with chemotherapy and bevacizumab alone, so that the combination of two biologics is nowadays contraindicated, regardless of *KRAS* mutational status. No data are yet available about the comparison between the two biologics, so that at present, the assessment of *KRAS* mutations is mandatory not only for identifying candidates to anti-EGFRs but for the rational choice of the best therapeutic strategy for mCRC patients.

On the other hand, it clearly appears that only a percentage of patients with *KRAS* wild-type disease derive benefit from anti-EGFR-containing regimens, underlining the need to further refine patient selection by identifying alternative predictive factors of intrinsic resistance to be combined with *KRAS* mutational status. Moreover, those patients who evidently respond to anti-EGFR monoclonal antibodies, often become rapidly resistant to the treatment, pointing out the occurrence of still unknown mechanisms of acquired resistance.

This paper will briefly review the following:

(1) The stages that have led to the definitive acquisition of *KRAS* assessment as an essential tool for the selection of patients candidate to receive anti-EGFR monoclonal antibodies.
(2) The state-of-the-art about other potential markers of intrinsic resistance.
(3) Preclinical evidence and future perspectives on markers of acquired resistance and potential strategies to overcome it.

KRAS mutations assessment: clinical evidence and technical issues

The first efforts to detect molecular factors able to predict the activity of anti-EGFR monoconal antibodies focused on EGFR, failing to demonstrate a correlation between the expression of the molecular target, as detected by immunohistochemistry (IHC) and drug activity [Hebbar *et al.* 2006; Cunningham *et al.* 2004].

In order to explain this paradox, different hypotheses have been formulated. Technical issues have been raised – such as the storage time, possible problems deriving from tissue fixation [Atkins *et al.* 2004], the possibility to detect by IHC EGFR epitopes other than those bound by monoclonal antibodies [Chung *et al.* 2005] – as well as biological questions, such as, the discrepancy between EGFR expression in primary tumors and related metastases [Scartozzi *et al.* 2004].

However, despite the lack of correlation between EGFR expression by IHC and clinical outcome, current regulatory restrictions still impose administration of anti-EGFR monoclonal antibodies only to patients with tumors that express EGFR as detected by IHC [Anon, 2008]. Also results obtained by fluorescent or chromogenic *in situ* hybridization [Sartore-Bianchi *et al.* 2007; Lievre *et al.* 2006; Moroni *et al.* 2005] appear hardly reproducible, due to the heterogeneity of adopted cut-offs and to the lack of a standardized procedure.

The attention has been, thus, focused on intracellular mediators of EGFR signaling. Several retrospective studies [Lievre *et al.* 2008; Benvenuti *et al.* 2007; De Roock *et al.* 2007; Di Fiore *et al.* 2007; Khambata-Ford *et al.* 2007; Lievre *et al.* 2006; Moroni *et al.* 2005] addressed the question of whether *KRAS*

mutations could predict the outcome of mCRC patients treated with EGFR inhibitors. Lièvre and colleagues [Lievre *et al.* 2006] reported for the first time that *KRAS* codon 12 and 13 mutations predict resistance to cetuximab in a series of 30 mCRC patients. Last year, the same authors [Lievre *et al.* 2008] confirmed their findings in a series of 89 patients treated with cetuximab: no responders were found among mutated patients *versus* 34 responders among wild-type patients ($p < 0.001$). PFS and overall survival (OS) were significantly longer in patients with *KRAS* wild-type tumors (median PFS: 10.1 *versus* 31.4 weeks, $p = 0.0001$; median OS: 14.3 *versus* 10.1 months, $p = 0.026$).

Recently, *post-hoc* analyses of final results of international phase III randomized trials, evaluating the role of anti-EGFR antibodies in the treatment of mCRC, have further ascertained the predictive power of *KRAS* mutational status.

In the CRYSTAL (Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer) study [Van Cutsem *et al.* 2009] patients with EGFR-expressing mCRC have been randomized to a first-line FOLFIRI regimen plus cetuximab or FOLFIRI alone. The addition of cetuximab significantly improved both PFS (8.9 *versus* 8.0 months, HR = 0.851, $p = 0.0479$) and response rate (RR, 46.9 *versus* 38.7%, $p = 0.005$). The subgroup analysis of final results in the light of the knowledge of *KRAS* mutational status showed that mainly patients with *KRAS* wild-type disease derived a significant advantage both in terms of PFS (9.9 *versus* 8.7 months, HR = 0.68, $p = 0.017$) and RR (59.3 *versus* 43.2%, OR = 1.91), by the administration of cetuximab combined with chemotherapy. Although the interaction between treatment group and *KRAS* mutational status was of borderline significance for PFS ($p = 0.07$), such results are, however, strongly suggestive of *KRAS* predictive value, and the statistical significance should be counterweighted with the great amount of data deriving from other series strongly supporting the absence of benefit for *KRAS* mutated patients. The OPUS (Oxaliplatin and Cetuximab in first-line treatment of mCRC) study [Bokemeyer *et al.* 2009] was a randomized phase II trial assessing the efficacy of FOLFOX plus cetuximab (*versus* FOLFOX) as first-line regimen in EGFR-expressing mCRC patients. RR was higher in the group of patients treated with the combination of chemotherapy and cetuximab (RR: 45.6 *versus* 35.7%, $p = 0.063$), without any difference in terms of PFS (7.2 *versus* 7.2 months, HR = 0.93, $p = 0.617$). The *post-hoc* analysis of results according to *KRAS* mutational status showed that among patients with *KRAS* wild-type disease, those treated with cetuximab experienced a better outcome both in terms of RR and PFS, in comparison with patients who had received only FOLFOX (RR: 60.7 *versus* 37.0%, $p = 0.011$; PFS: 7.7 *versus* 7.2 months, HR = 0.57, $p = 0.016$).

When compared to best supportive care (BSC) in advanced chemorefractory disease, both cetuximab and panitumumab demonstrated a survival benefit restricted to patients with *KRAS* wild-type tumors. Van Cutsem and colleagues [Van Cutsem *et al.* 2007] published the first report that panitumumab significantly prolonged PFS in chemorefractory mCRC, when compared to BSC (HR = 0.54, $p < 0.0001$). Subsequently, Amado and colleagues [Amado *et al.* 2008] demonstrated that the advantage from panitumumab was confined to *KRAS* wild-type patients (median PFS: 12.3 *versus* 7.3 weeks, HR = 0.45) and concluded that the absence of *KRAS* mutations was required for panitumumab efficacy. The CO.17 study [Jonker *et al.* 2007] investigated the efficacy of cetuximab monotherapy compared to BSC in EGFR-expressing mCRC patients, who had previously failed fluoropyrimidine-, irinotecan- and oxaliplatin-based therapies. Cetuximab significantly reduced the risk of disease progression (HR = 0.68, $p < 0.0001$) and improved OS (6.1 *versus* 4.6 months, HR = 0.77, $p = 0.005$). Karapetis *et al.* analyzed *KRAS* mutational status in this population, reporting again that, only in *KRAS* wild-type patients, cetuximab significantly improved PFS (3.7 *versus* 1.9 months, HR = 0.40, $p < 0.001$) and OS (9.5 *versus* 4.8 months, HR = 0.55, $p < 0.001$) [Karapetis *et al.* 2008]. Moreover, the authors suggested that *KRAS* mutations have no influence on survival among patients treated with BSC alone, underlining their predictive, more than prognostic, impact.

Moving from the above reported results of *post-hoc* analyses and retrospectively collected series, the use of monoclonal antibodies is now restricted to patients with *KRAS* wild-type disease [Allegra *et al.* 2009].

It should be noted that outcome data according to *KRAS* mutational analysis have been provided

also for both CAIRO-2 [Tol *et al.* 2009a] and PACCE [Hecht *et al.* 2009] trials, with the aim to evaluate the addition of anti-EGFRs to bevacizumab plus chemotherapy, even if the trials were already ongoing while data on *KRAS* were becoming more and more clear. Patients bearing *KRAS* wild-type tumors do not seem to benefit from the addition of the anti-EGFR antibody to the combination of chemotherapy and bevacizumab, thus not supporting VEGF/EGFR double inhibition even in this subpopulation, although such considerations derive from secondary analyses that have limited power.

From a technical perspective, important questions are raised by the implementation of *KRAS* analysis in clinical practice. Indeed, different techniques have been chosen in different studies in order to detect *KRAS* mutations and there is not a unique indication for a specific test to be routinely adopted. It has been pointed out that it is necessary to define and validate all the steps of the procedure, keeping in mind the optimal balance between accuracy and practicality as a requirement that cannot be renounced [Jimeno *et al.* 2009]. First of all, it would be important to define a sufficient tumor DNA quantity, suggested as a minimum of 30 μg of template DNA, an amount easily obtained from formalin-fixed paraffin embedded tissue blocks, which are the most clinically available sources. Second, in order to avoid the dilution of tumor DNA with that of reactive cells around the tumor, it is important to validate a form of tumor cell enrichment as micro- or macro-dissection or selective sampling by needle core. Finally, it would be necessary to better define the testing procedure itself, considering that several methods have been described: restriction fragmentation length polymorphism (RFLP); allele specific oligonucleotide (ASO) hybridization [Van Heek *et al.* 2005]; high-resolution melting analysis (HMRA) [Simi *et al.* 2008]; amplification refractory mutation system (ARMS) [Van Heek *et al.* 2005], and new technologies such as pyrosequencing are under development [Ogino *et al.* 2005].

Nowadays, both direct sequencing and real-time PCR-based assays have demonstrated themselves as reliable tests, with a sensitivity of 95.5 and 96.5%, respectively [Tol *et al.* 2009b]. Due to its higher sensitivity, real-time PCR is the best choice to be used in samples with poor cellularity.

The effort to standardize procedures is still a major objective. National and international quality assurance programs have been put into action and are currently ongoing, with the aim to guarantee homogenous evaluations as far as possible [Van Krieken *et al.* 2009].

In conclusion, *KRAS* testing is progressively changing the approach to mCRC management for both oncologists and pathologists. Nevertheless, since a consistent group of patients with *KRAS* wild-type tumors do not derive benefit from EGFR inhibitors, additional reliable and easily measurable biomarkers are needed to further improve the selection of patients, increase the cost effectiveness of treatment and avoid unnecessary toxicity. In fact, in a systematic review and meta-analysis, Linardou and colleagues [Linardou *et al.* 2008] reported a very high specificity (0.93, [0.83–0.97]), but a much lower sensitivity (0.47, [0.43–0.52]) of *KRAS* analysis in predicting resistance to EGFR inhibitors in mCRC patients, underlining the need to investigate new potential biomarkers, beyond *KRAS* codon 12 and 13 mutations.

Perspectives on new potential markers of activity of EGFR inhibitors in mCRC

Beyond KRAS: genotyping tumors

KRAS-activating mutations, other than those affecting codon 12 and 13, have been described in mCRC. These relatively rare mutations, involving codons 61 and 146 [Edkins *et al.* 2006], have been detected with frequencies ranging from 1 to 4% and determine the constitutive activation of RAS protein, due to its reduced GTPase activity or to its increased affinity for GTP [Buhrman *et al.* 2007]. These mutations are mutually exclusive with codon 12 and 13, and might represent another potential marker of resistance to anti-EGFR antibodies in *KRAS* codon 12 and 13 wild-type patients. Moreover, RAS protein is encoded not only by *KRAS*, but also by other homologous genes belonging to the so called 'RAS superfamily', *NRAS* and *HRAS* [Rodriguez-Viciana *et al.* 2005].

The analysis of a series of 572 paraffin-embedded CRC samples, tested for *KRAS*, *NRAS*, *HRAS* codon 12, 13 and 61 mutations, has recently revealed that up to 11% of RAS mutations would be missed if only codons 12 and 13 of *KRAS* had been analyzed [Albitar *et al.* 2009].

Similarly, among 108 *KRAS* codon 12 and 13 wild-type samples, *NRAS*-activating mutations were found in five cases (about 5%), and none of the patients with *NRAS*-mutated disease achieved response to cetuximab [De Roock *et al.* 2009].

Due to the relatively rare occurrence of these activating mutations, adequately powered studies are required to further investigate their role in improving selection of patients for therapy.

The disregulation of the RAS/RAF/MAPK pathway also occurs as a result of the constitutive activation of RAF protein. *BRAF* V600E mutation induces structural changes in RAF protein, thus increasing its kinase activity [Wan *et al.* 2004]. About 10% of CRCs bear this mutation, which is mutually exclusive with *KRAS* ones [Rajagopalan *et al.* 2002]. Benvenuti and colleagues [Benvenuti *et al.* 2007] produced a first report of 48 mCRC patients treated with anti-EGFR monoclonal antibodies showing that the presence of *KRAS* and/or *BRAF* mutations was negatively associated with response ($p = 0.005$). The same authors recently demonstrated in a cohort of 79 *KRAS* wild-type patients that *BRAF* V600E mutation was correlated with resistance to cetuximab and panitumumab administered either alone or in combination with chemotherapy [Di Nicolantonio *et al.* 2008]. None of 11 (13%) patients with *BRAF*-mutated tumors responded to the treatment, whereas none of the responders carried the mutation ($p = 0.029$). *BRAF* mutation was also associated with shorter PFS ($p = 0.0010$) and OS ($p = 0.0001$). These results were confirmed by *in vitro* assays, demonstrating that, while *BRAF*-mutated HT-29 and COLO-205 lines were highly refractory to anti-EGFR monoclonal antibodies, *BRAF* wild-type DiFi cells were inhibited by nanomolar drug concentrations.

As previously described, RAS and RAF belong to the same signaling cascade, representing only one side of the axis on which EGFR relies to propagate its mitogenic stimulus. Another important signaling pathway is the one of PTEN/PI3K/Akt. PI3K is a lipid kinase, encoded by the *PIK3CA* gene, whose activity is normally balanced by PTEN. *PIK3CA* is mutated in about 20% of mCRC patients [Samuels *et al.* 2004] in the hotspots located in exons 9 and 20. An *in vitro* study has suggested that the dysregulation of the PTEN/PI3K/AKT pathway, as a result of *PIK3CA*-activating mutations or loss of PTEN expression, may predict response of CRC cell lines to cetuximab [Jhawer *et al.* 2008]. Moreover, cell lines with activating alterations of both RAS/RAF/MAPKs and PTEN/PI3K/Akt pathways were more resistant to cetuximab than cell lines harboring only one altered pathway.

Early studies [Lievre *et al.* 2006; Moroni *et al.* 2005] conducted in small series of mCRC patients treated with anti-EGFRs failed to demonstrate a significant correlation between *PIK3CA* mutations and resistance to treatment. Subsequently, Perrone and colleagues [Perrone *et al.* 2008] showed in a cohort of 32 mCRC patients treated with cetuximab that PTEN/PI3K deregulation (as a direct consequence of *PIK3CA* mutations or as an indirect result of the loss of PTEN phosphatase activity) significantly correlates with an impaired response to cetuximab ($p = 0.02$). Recently, Sartore-Bianchi and colleagues [Sartore-Bianchi *et al.* 2009] evaluated the impact of *PIK3CA* mutations in a cohort of 110 mCRC patients treated with cetuximab or panitumumab, administered alone or in combination with chemotherapy. *PIK3CA* mutations, found in 13.6% of cases, were significantly associated with a lack of response to anti-EGFRs, with none of patients with the mutation achieving response ($p = 0.038$). In the *KRAS* wild-type population, the correlation between *PIK3CA* mutations and resistance to anti-EGFRs was confirmed, both in terms of RR ($p = 0.016$) and PFS ($p = 0.0021$), leading the authors to hypothesize that the combination of both analyses could allow the identification of a higher percentage of patients resistant to cetuximab or panitumumab.

Conversely, Prenen and colleagues [Prenen *et al.* 2009] found no correlation between *PIK3CA* mutations and impaired clinical outcome, either in terms of RR ($p = 0.781$), or in PFS ($p = 0.760$), or in OS ($p = 0.698$), in a series of 200 patients treated with cetuximab alone or in combination with irinotecan. Also, *PIK3CA* mutations were not associated with lower RR in the subgroup of patients with *KRAS* wild-type disease ($p = 0.758$).

The lack of conformity among these results points out the need for adequately dimensioned trials, in order to conclusively address the item of the potential role of *PIK3CA* alterations as determinants of intrinsic resistance.

Moreover, since at present the knowledge about the simultaneous occurrence of *BRAF* and *PIK3CA* genetic alterations is extremely poor – and it rather seems that they are not mutually exclusive [Ogino *et al.* 2009] – it would be interesting to assess both alterations in the same population, in order to clarify their impact as independent predictors of resistance.

A recent retrospective study conducted on 64 mCRC patients who had received salvage cetuximab plus irinotecan, would suggest that *TP53* exons 5–8 mutations might predict sensitivity to the treatment, both in terms of disease control rate (DCR, $p = 0.037$) and time-to-progression (TTP, $p = 0.004$). Similar results were reported in the subgroup of 46 patients with *KRAS* wild-type tumors [Oden-Gangloff *et al.* 2009]. These findings are surprising taking into account the negative prognostic value of *TP53* in stage II and III CRCs [Westra *et al.* 2005]. The authors explain such results by referring to the role of p53 as 'policeman of oncogenes'. According to their interpretation, the loss of p53 activity that results in the loss of a brake for hyperactive intracellular pathways downstream EGFR, would exasperate the crucial oncogenic role of such pathways. These tumors would be remarkably dependent on EGFR, so that EGFR inhibition would represent a particularly effective strategy to control their growth.

However, further efforts are needed to corroborate or deny this hypothesis. In particular, the complex function of p53 at the crossroads of multiple cellular response pathways, other than EGFR ones that strongly determine cellular fate should be considered. Moreover, future studies should also consider the functional connection between p53 and Mdm2, that regulates p53, both reducing its transcriptional activity and increasing its ubiquitination rate [Piette *et al.* 1997].

Beyond KRAS: 'genotyping patients'

About 10 million single-nucleotide polymorphisms (SNPs) have been described throughout human genomic DNA. SNPs of genes involved in the EGFR pathway may lead to aberrant EGFR activation, determining resistance to anti-EGFR treatment through different mechanisms. The possibility to identify genetic variants and the ability to predict sensitivity to anti-EGFRs, would be very attractive, considering that such assays can be easily performed in normal tissue (such as blood cells) with simple and standardized techniques.

A highly polymorphic sequence repeat of CA dinucleotide has been described in EGFR intron – 1 [Amador *et al.* 2004]. Experimental models revealed that EGFR transcriptional activity and EGFR expression are influenced by the number of CA repeats. In particular, an increase in the number of CA tandem repeats is associated with inferior levels of mRNA and protein expression [Gebhardt *et al.* 1999]. Graziano and colleagues [Graziano *et al.* 2008] studied EGFR intron 1 genetic variants by means of PCR and separation with capillary electrophoresis in a cohort of 110 patients with mCRC, treated with cetuximab and irinotecan. Considering the distribution of the EGFR intron-1 $(CA)_n$ repeats alleles in the Caucasian population, the authors defined EGFR intron-1 as either short (S) or long (L) when the number of CA repeats was $<$ or ≥ 17 respectively, thus distinguishing three possible genotypes: S/S, S/L or L/L. A significant correlation was found between EGFR intron-1 S/S variant and favourable OS. In this experience, *KRAS* mutational status was not assessed, so that the real predictive/prognostic implication of EGFR intron-1 polymorphism in *KRAS* wild-type populations was not clarified.

On the other hand, Lurje and colleagues [Lurje *et al.* 2008] did not find any correlation between EGFR intron 1 CA repeats and clinical outcome, identifying 20 CA repeats as the cut-off value to discriminate allelic variants determined by PCR and direct sequencing. New studies are needed to standardize techniques and cut-off values, while taking into account discrepancies in allelic frequencies in different populations.

EGF 61 A/G polymorphism may influence EGF transcription. In particular *EGF* 61 G allele is associated with a more elevated transcriptional level of EGF in comparison with *EGF* A variant [Shahbazi *et al.* 2002]. Graziano and colleagues reported a significant correlation between *EGF* 61 G/G genotype and favourable OS. More recently, two studies [Garm Spindler *et al.* 2009; Lurje *et al.* 2008] did not confirm the potential role of the *EGF* 61 G/G genotype in predicting the clinical outcome of mCRC patients treated with cetuximab.

In particular, Lurje and coworkers studied, in a cohort of 130 patients, 11 polymorphisms within

8 genes involved in the EGFR pathway, showing that EGFR pathway-related polymorphisms might be important prognostic markers, regardless of *KRAS* mutational status. They also found a significant association among *EGFR* 497 G > A, cyclo-oxygenase (COX)-2 765 G > C, *COX-2* 8473 T > C polymorphisms and favourable OS. While the relationship between COX-2 enzyme, induced by several cytokines and growth factors, and the EGFR signaling pathway is still controversial [Xu and Shu, 2007], *EGFR* 497 G > A polymorphism causes an amino-acidic substitution in the extracellular domain, which confers an attenuated function in EGFR-ligand binding, growth stimulation and tyrosine kinase activation [Moriai *et al.* 1994].

Finally, monoclonal antibodies partially exert their anti tumoral activity by recruiting cytotoxic host effector cells through the immunological process known as antibody-dependent cell-mediated cytotoxicity (ADCC). *In vitro* studies have shown that cetuximab [Kawaguchi *et al.* 2007] is able to induce ADCC. Polymorphisms of genes encoding the activating receptors *FcγIIa* and *FcγIIIa*, expressed on macrophages and natural killer cells, might affect their affinity for monoclonal antibodies [Niwa *et al.* 2004; Van Royen-Kerkhof *et al.* 2004].

Four retrospective studies have investigated the predictive/prognostic implication of *FcγIIa*-131 H/R and *FcγIIIa*-158 V/F polymorphisms in mCRC patients treated with cetuximab [Bibeau *et al.* 2009; Graziano *et al.* 2008; Lurje *et al.* 2008; Zhang *et al.* 2007]. While Graziano and colleagues did not find any correlation between FcγR investigated polymorphisms and clinical outcome, Zhang and colleagues suggested a potential role for both *FcγIIa*-131 A/A and *FcγIIIa*-158 V/V polymorphisms as unfavourable predictive factors in patients treated with single agent cetuximab. In a subsequent larger series by the same authors, these significant correlations were not found [Lurje *et al.* 2008]. Recently, Bibeau and coworkers [Bibeau *et al.* 2009] reported that patients with *FcγIIa*-131 H/H and/or *FcγIIIa*-158 V/V genotypes had longer PFS and OS, but not higher RR, than carriers of 131R and 158F variants. The same correlation was observed both in patients with *KRAS* wild-type and *KRAS*-mutated tumors, thus not excluding a prognostic significance for such variants. Therefore, available results, in particular as it involves *FcγIIIa*-158 V/F polymorphism, appear remarkably conflicting.

Although reliable and easily assessable in clinical practice, candidate polymorphisms have so far failed to demonstrate a clear predictive potential and it seems rather unlikely to translate such discordant results into clinical practice.

Beyond genetic features: 'phenotyping tumors'

Multiple pieces of evidence have proven the importance of the tissue microenvironment in conditioning the multiple steps of malignant transformation, tumoral progression and cellular survival and proliferation. For this reason, it is plausible that not only genotypic but also phenotypic features may interfere with tumoral sensitivity to anti-EGFR antibodies.

PTEN is a dual-specificity phosphatase whose major substrate is represented by PIP3. PTEN counteracts PI3K kinase activity, diminishing the activation of the PI3K/Akt pathway. As well as genetic alterations (e.g. allelic deletions, point mutations and loss of heterozigosity) epigenetic mechanisms may also compromise PTEN functioning [Goel *et al.* 2004]. Moreover, it has been shown that all cited alterations result in the reduction of PTEN expression [Zhou *et al.* 2002]. For this reason, IHC might provide an appreciation of PTEN aberrations better than techniques that investigate only genetic features.

The potential relation between PTEN loss, as detected by IHC on primary tumors, and resistance to anti-EGFRs has been suggested in a small series of 27 patients, treated with cetuximab alone or associated with chemotherapy, where none of 11 patients with loss of PTEN responded in comparison with 10 out of 16 patients in which the protein was normally expressed ($p < 0.001$) [Frattini *et al.* 2007]. It should be noticed that in this retrospective series, cetuximab was administered in combination with different chemotherapy regimens and in different lines of treatment. The heterogeneity of adopted schemes and the inclusion in the study population of patients receiving very active first-line regimens, such as capecitabine plus oxaliplatin, in association with cetuximab, makes it difficult to evaluate the real contribution of EGFR inhibition to the observed responses.

In our recent series, comprising 102 patients treated with the combination of cetuximab and

irinotecan, PTEN-IHC, successfully performed on 85 primary tumors, was not able to predict the treatment outcome. Nevertheless, it was shown a significant discrepancy between primary tumors and related metastases, as already observed with regard to other signal transductors (i.e., EGFR and MAPKs) [Loupakis *et al*. 2009]. Among 45 available pairs, IHC results were concordant only in 27 instances (60%; 95% CI, 46–74%; $p = 0.346$). When the analysis was performed on samples from metastases, loss of PTEN-IHC was significantly related with resistance to the treatment, in terms of both RR (5 *versus* 26%; $p = 0.007$) and PFS (3.3 *versus* 4.7 months; $p = 0.005$). Also, in the subgroup of patients with *KRAS* wild-type disease, loss of PTEN was correlated with significantly shorter PFS (3.7 *versus* 5.3 months; $p = 0.026$), confirming the potential implication of such determination in the prediction of benefit from anti-EGFRs [Loupakis *et al.* 2009]. Even if quite appealing, the exploratory nature of these retrospective analyses cannot be forgotten, as well as the limitations of PTEN-IHC as an unvalidated technique.

Attention has been focused also on the expression levels of EGFR endogenous ligands and in particular amphiregulin (AR) and epiregulin (ER). Khambata-Ford and colleagues have first observed that patients with tumors expressing elevated transcriptional levels of AR and ER specific mRNAs were more likely to experience disease control when treated with cetuximab monotherapy (ER, $p = 0.000015$; AR, $p = 0.000025$) [Khambata-Ford *et al.* 2007]. They also observed the lack of correlation between tumoral mRNA expression and plasma-protein levels, which leads to consider the hypothetical existence of complex post-transcriptional regulatory mechanisms. Similarly, in the series presented by Tejpar and coworkers higher levels of AR and ER specific mRNAs, assessed by RT-PCR in tumoral tissues, were reported among responding patients, in comparison with non responders ($p < 0.0001$) [Tejpar *et al.* 2008].

Also, as it involves EGFR endogenous ligands, further investigations addressing technical and biological issues are needed to really understand the predictive/prognostic implications of such determinations, bearing in mind the not negligible influence that *KRAS* mutations have on the activity of anti-EGFR monoclonal antibodies.

In conclusion, relevant methodological limits affect not only the search for new predictive biomarkers, but also the routine adoption of *KRAS* mutations assessment in clinical practice. The lack of validated techniques and standardized methods, the necessarily retrospective nature of collected data and the 'hypothesis-generating' more than 'hypothesis-generated' logic of many experiences should be borne in mind when interpreting certain 'promising' results. Thus, caution is imperative when interpreting results of small retrospective experiences that could be influenced by inadequate sample size. Moreover, from now on, since the use of anti-EGFR monoclonal antibodies is restricted to patients bearing *KRAS* wild-type tumors, the effort to realize adequately powered clinical studies will faced with the necessity to include only 60% of screened patients who represent the population actually candidate to the treatment.

From a statistical point of view, the difficulty to design well-dimensioned trials is further complicated by the heterogeneous expression of many investigated biomarkers in tumors, due to the emergence of clonal populations in the natural history of neoplastic diseases. As well as technical errors, inherent heterogeneity may impair the reported effect size and thus compromise the power of a study [Pintilie *et al.* 2009]. For this reason, it would be desirable, if not imperative, to conduct pilot studies aiming to estimate the heterogeneity of a candidate marker within and between individuals, and to use the knowledge coming from these exploratory preambles to appropriately design translational trials.

Future perspective: potential markers of acquired resistance

Although anti-EGFR therapies may be initially effective, clinicians know from clinical practice that neoplastic diseases will become rapidly refractory to such treatment in the great majority of treated patients. In order to better understand the mechanisms leading to acquired resistance in patients with initially sensitive tumors and to identify potential strategies to overcome resistance, a substantial advance is awaited from translational research.

At present, no conclusive data have emerged from the literature. Even if some preclinical studies addressed the issue of acquired resistance, the body of evidence is much less extensive compared with the large amount of experience aimed

at identifying predictive factors of intrinsic resistance. To date, two main mechanisms have been hypothesized as mediators of acquired resistance:

(1) the hyperfunction of alternative growth pathways (mainly VEGF), through which tumoral cells might escape the blockade of EGFR
(2) the constitutive activation of downstream effectors, involved in different pathways as signal transducers.

Escaping from EGFR blockade: the implications of the VEGF pathway

It has been ascertained that the inhibition of tumor angiogenesis is one of the biological effects of EGFR inhibition and may significantly contribute to the antitumor efficacy of anti-EGFR monoclonal antibodies. Several studies have reported a decreased secretion of VEGF and other pro-angiogenic growth factors, including basic fibroblast growth factor (bFGF), interleukin-8 (IL-8) and transforming growth factor-*α* (TGF-*α*), as a result of EGFR inhibition [Petit *et al.* 1997]. The reduced expression of angiogenic factors leads to a significant decrease in microvessel density and to an enhanced apoptotic stimulus in human tumor xenografts [Bruns *et al.* 2000; Perrotte *et al.* 1999].

Consistently with these considerations, different preclinical experiences (*in vitro* and *in vivo)* showed significant increases in VEGF expression as detected by ELISA assay and IHC, respectively performed on the conditioned medium of cell cultures and on sections of human xenografts, which had become resistant to EGFR inhibitors after an initial response.

Viloria-Petit and colleagues firstly described a twofold increase in VEGF protein and mRNA in five of six variants of the human A431 squamous cell carcinoma cell line, derived from xenografts which had become resistant to cetuximab. IHC staining of sections from resistant xenografts revealed the presence of a number of conspicuously large and immature blood vessels and a higher overall amount of tissue VEGF [Viloria-Petit *et al.* 2001].

Similarly, Ciardiello and colleagues observed that long-term treatment of human GEO colon cancer xenografts with cetuximab or ZD1839 (gefitinib) resulted in the development of resistant cell lines, which exhibited a 5–10 fold increase of the expression of VEGF, COX-2, and of the phosphorylated, activated forms of mitogen-activated protein kinases (pMAPKs) [Ciardiello *et al.* 2004]. Conversely, in resistant GEO cells, EGFR expression appeared only slightly reduced in comparison with parental sensitive lines. Interestingly, the treatment of human GEO xenografts with the multitargeted tyrosine kinase inhibitor ZD6474 (vandetanib), binding both VEGFR1-3 and EGFR, did not lead to the emergence of resistant tumors. Moreover, ZD6474 was able to control the growth of GEO xenografts that had become resistant to anti-EGFR drugs; thus, pointing out the role of sequential targeting of multiple oncogenic pathways as an appealing strategy to be attempted in future preclinical and clinical trials. The same authors subsequently observed a noticeable increase in VEGFR-1 expression in resistant GEO cells, which also synthesized and secreted much higher levels of VEGFR ligands, VEGF and placental growth factor (PlGF), compared with parental lines [Bianco *et al.* 2008]. The interruption of the established autocrine loop, through siRNA silencing of VEGFR-1, partially restored sensitivity to anti-EGFR drugs and impaired migration efficiency and thus metastatic potential sustained by VEGFR-1 hyperactivity. Similar effects have been described as a result of the inhibition of the signaling pathway downstream VEGFR. Enzastaurin is a potent serine/threonine kinase inhibitor, whose anti-angiogenic efficacy is due to the inhibition of the protein kinase C (PKC) isoform β(PKCβ), which mediates the transduction to the nucleus of the VEGFR signal [Faul *et al.* 2003]. Enzastaurin was able to restore the sensitivity to gefitinib of gefitinib-resistant GEO cell lines and xenografts, when administered both alone and, more efficaciously, in combination with gefitinib [Gelardi *et al.* 2008].

The involvement of the VEGF pathway as a mechanism of acquired resistance to anti-EGFR drugs has been recently strengthened by the experience of Benavente and coworkers who demonstrated a marked increase of angiogenic potential of cetuximab-, gefitinib- or erlotinib-resistant SCC-1 cells, subcutaneously implanted in Matrigel plugs into nude mice [Benavente *et al.* 2009]. In comparison with parental lines, plugs containing resistant cells showed extensive vascularization and aberrant growth of blood vessels toward the tumor core.

Less numerous evidence supports the involvement of the insulin-like growth factor-1 receptor (IGF-1R) pathway [Chakravarti *et al.* 2002] and HER family members [Wheeler *et al.* 2008] in overriding the effect of EGFR inhibitors and, thus, in contributing to the emergence of acquired resistance.

Escaping from EGFR blockade: the implication of EGFR and its downstream signaling mediators

An unanswered question still remains regarding the role of EGFR expression and functioning as a mechanism of acquired resistance. Viloria-Petit and colleagues reported that cell lines established from resistant xenografts retained EGFR expression levels similar to those of parental lines [Viloria-Petit *et al.* 2001]. Following TGF-α stimulation, the level of EGFR phosphorylation in resistant cell lines was not different from that of parental lines, thus suggesting an unaltered sensitivity of EGFR to its ligands.

On the other hand, a significant reduction of EGFR levels, as detected by Western blot analysis, has been described by Lu and colleagues in cetuximab-resistant DiFi5 CRC cell lines (in comparison with parental lines), probably due to a dramatic increase of EGFR ubiquitination, which would alter the trafficking and the expression of the receptor. Although EGFR expression levels were relevantly lower, DiFi5 cells exhibited higher levels of phosphorylated EGFR and Akt in response to EGF stimulation [Lu *et al.* 1997].

Preclinical data also suggest the implication of Src family kinases in the emergence of resistance [Wheeler *et al.* 2009]. The oncogenic collaboration between Src kinases and EGFR in malignant transformation has been well defined in earlier studies: high levels of Src kinases would potentiate the EGFR-mediated mitogenic stimulus [Biscardi *et al.* 1999; Tice *et al.* 1999]. Src kinase inhibitors, dasatinib and PP2, were able to partially restore the sensitivity to cetuximab of both colorectal and lung cancer resistant cell lines, thus suggesting the cooperation of EGFR and Src kinases as a mechanism of acquired resistance to EGFR inhibitors [Wheeler *et al.* 2009; Lu *et al.* 1997].

To date, preclinical data about the modulation of intracellular signaling mediators are quite ambiguous. Ciardiello and colleagues saw no evidence of major changes in levels of signal transducers [Ciardiello *et al.* 2004]; however, other reports attribute a relevant role to the constitutive activation of pathways downstream EGFR. Benavente and colleagues have shown that in resistant cells the administration of anti-EGFR drugs is not followed by the expected reduction of the activated forms of signal transducers, such as phosphorylated MAPKs, STAT3 and Akt, as observed in parental lines [Benavente *et al.* 2009].

Particular attention has been paid to PI3K/Akt/PTEN pathway and its downstream effectors, such as the mammalian target of rapamicin (mTOR)/p70S6K complex. Bianco and colleagues have observed that, while cetuximab and gefitinib did not determine a significant reduction of phosphorylated Akt and p70S6K in resistant cells, the partial reversion of resistance achieved by vandetanib, was accompanied by a reduction of activated mediators to levels not different from those observed in sensitive cells treated with anti-EGFRs [Bianco *et al.* 2008]. Similarly, the efficacy of dasatinib in the control of resistant cell growth and survival was accompanied by a reduction of phosphorylated Akt levels. The hyperactivity of Akt/mTOR pathway as a potential mechanism of resistance to anti-EGFRs is, moreover, confirmed by the ability of the mTOR inhibitor everolimus to restore the sensitivity to gefitinib and cetuximab of resistant GEO cells and xenografts [Bianco *et al.* 2008].

Even if it is extremely appealing and certainly promising, the hypothesis of the inhibition of multiple signaling pathways is still far from being rapidly adopted in clinical practice. Despite encouraging preclinical evidence, large phase III randomized trials have failed, as already mentioned, to demonstrate an advantage from the combined inhibition of EGFR- and VEGF-pathways in first-line treatment of patients with mCRC [Hecht *et al.* 2009; Tol *et al.* 2009a].

Conversely, the potential efficacy of sequential schemes, introducing a target inhibitor with anti-angiogenic properties beyond progression to an anti-EGFR treatment, certainly deserves further prospective study.

Conclusion

The inhibition of EGFR represents an effective strategy in the treatment of mCRC. The identification of *KRAS*-activating mutations as predictors of resistance to treatment with anti-EGFR monoclonal antibodies and, consequently, the

restriction of the use of these drugs to patients with *KRAS* wild-type disease, marks a crucial turning-point in the treatment of mCRC, since for the first time, therapeutic choices are deeply conditioned by a molecular determinant. On the other hand, non-negligible methodological and logistical problems arise from the routine adoption of *KRAS* assessment in clinical practice, as, first of all, the need for validated and standardized assays.

The search for alternative predictive factors of intrinsic resistance, to be integrated with the well ascertained determination of *KRAS* mutations, will further influence patient selection for treatment, but the pathway from retrospective evidence to the final translation in clinical practice is often winding.

Daily clinical experience shows that patients initially responsive to anti-EGFR drugs often become rapidly resistant to the treatment; thus, acquired resistance is another major issue to be urgently faced. The intriguing hypothesis of the contemporary or sequential inhibition of multiple pathways as a strategy to override resistance will certainly deserve further investigation. To this end, the early identification of molecular markers of outcome, might help to optimize the selection of patients to be subsequently included in appropriately designed clinical trials.

Conflict of interest statement

None declared.

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