KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer

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BACKGROUND: KRAS codons 12 and 13 mutations predict resistance to anti-EGFR monoclonal antibodies (moAbs) in metastatic colorectal cancer. Also, BRAF V600E mutation has been associated with resistance. Additional KRAS mutations are described in CRC.

METHODS: We investigated the role of KRAS codons 61 and 146 and BRAF V600E mutations in predicting resistance to cetuximab plus irinotecan in a cohort of KRAS codons 12 and 13 wild type patients.

RESULTS: Among 87 KRAS codons 12 and 13 wild type tumours, KRAS codons 61 and 146 were mutated in 7 and 1 case, respectively. None of mutated patients responded vs 22 of 68 wild type (P = 0.096). Eleven patients were not evaluable. KRAS mutations were associated with shorter progression-free survival (PFS; HR: 0.46, P = 0.028). None of 13 BRAF-mutated patients responded vs 24 of 74 BRAF wild type (P = 0.016). BRAF mutation was associated with a trend towards shorter PFS (HR: 0.59, P = 0.073). In the subgroup of BRAF wild-type patients, KRAS codons 61/146 mutations determined a lower response rate (0 vs 37%, P = 0.047) and worse PFS (HR: 0.45, P = 0.023). Patients bearing KRAS or BRAF mutations had poorer response rate (0 vs 37%, P = 0.0005) and PFS (HR: 0.51, P = 0.006) compared with KRAS and BRAF wild-type patients.

CONCLUSION: Assessing KRAS codons 61/146 and BRAF V600E mutations might help optimising the selection of the candidate patients to receive anti-EGFR moAbs.

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RAS and RAF proteins play a key role in the control of cellular growth, proliferation and differentiation (Bos, 1989; Wickenden et al, 2008) KRAS-activating mutations reduce or abolish intrinsic GTPase activity of the protein, leading to its constitutive activation (Conlin et al, 2005) Similarly BRAF V600E mutation induces structural changes in RAF protein which increase its kinase activity (Wan et al, 2004) Activated RAS and RAF are responsible for the disregulation of RAS/RAF/MEK/ERK signalling pathway.

KRAS codons 12 and 13 activating mutations are widely recognised as predictors of resistance to the treatment with anti-EGFR monoclonal antibodies (moAbs) in metastatic colorectal cancer (mCRC) patients (Karapetis et al, 2008; Amado et al, 2008). Based on retrospectively collected data and post hoc analyses of large phase III studies, the European Medicines Agency has restricted the use of cetuximab to the treatment of patients with KRAS codons 12 and 13 wild-type disease (about the 60% of the overall population; EMEA, 2008) and the American Society of Clinical Oncology has similarly recommended in a recent provisional clinic opinion not to administer anti-EGFR moAbs to patients with KRAS codons 12 or 13 mutated tumours (Allegra et al, 2009). Nevertheless, in a systematic review and meta-analysis, Linardou et al reported a very high specificity (0.93, (0.83–0.97)) and a much lower sensitivity (0.47, (0.43–0.52)) of KRAS analysis in predicting resistance to anti-EGFR moAbs in mCRC, thus underlining the need for additional predictive markers to be combined with KRAS codons 12 and 13 evaluation, for a more accurate patients’ selection (Linardou et al, 2008).

A recently published experience found a correlation between BRAF V600E-activating mutation, mutually exclusive with KRAS ones, and resistance to the treatment with cetuximab and panitumumab administered alone or in combination with chemotherapy (Di Nicolantonio et al, 2008).

On the basis of the above-mentioned evidences, around 50% of candidate patients would be excluded from receiving anti-EGFR...
moAbs, with a significant improvement of the treatment’s cost effectiveness. However, as cetuximab or panitumumab monotherapies determine a response rate of about 10% regardless of the line of treatment and no more than 23% of patients respond to the combination of cetuximab and irinotecan (Cunningham et al., 2004; Van Cutsem et al., 2007; Jonker et al., 2007) it is plausible that the selection of candidate patients to receive an anti-EGFR moAb might be further slightly refined.

Additional KRAS-activating mutations, involving codons 61 and 146 (Edkins et al., 2006) occur with frequencies ranging from 1 to 4% in CRCs. These relatively rare mutations, as well as codons 12 and 13 mutations, are responsible for the oncogenic constitutive activation (Buhrman et al., 2007; Feig and Cooper, 1988) of RAS/RAF/MEK/Erk pathway and they might account for up to a 10% of resistant patients bearing KRAS codons 12 and 13 and BRAF wild-type tumours.

To optimise the selection of patients who are more likely to benefit from anti-EGFR we investigated in a cohort of patients treated with the combination of cetuximab and irinotecan and bearing KRAS codons 12 and 13 wild-type tumours, the association of KRAS codons 61 and 146 mutations and BRAF V600E mutation with clinical outcomes.

MATERIALS AND METHODS

Patients

Patients with irinotecan-refractory mCRC (ie, progressed during or within 3 months after treatment with an irinotecan-based regimen) were considered eligible for our study if they had an histologically confirmed diagnosis of EGFR-positive adenocarcinoma, measurable and evaluable disease according to RECIST criteria (Therasse et al, 2000) available paraffin-embedded samples of primary lesions and had undergone a salvage cetuximab-irinotecan treatment.

All patients’ samples were screened for KRAS codons 12 and 13 mutations, constituting the group named ‘overall population’ and further analysed for the group named ‘study population’ and further analysed for mutations of primary lesions and had undergone a salvage cetuximab-irinotecan treatment.

Response was evaluated every 8 weeks by CT-scan. According to RECIST criteria, patients were categorised as responders if they achieved complete response (CR) or partial response (PR), or non-responders if they showed stable (SD) or progressive disease (PD).

Results of statistical analysis included the association between KRAS and BRAF mutational status and the primary end-point (categorical variables: presence or absence of the mutation). The primary end-point was the correlation between KRAS codon 61, 146, BRAF V600E mutations and response to treatment in the study population. Two-tailed Fisher’s exact test was used to compare proportions of responders and non-responders according to their mutational status. The PFS and OS analyses were determined according to the Kaplan–Meier method and survival curves were compared using the log-rank test. Statistical significance was set at P<0.05 for a bilateral test.

Table 1  Primers and polymerase chain reaction (PCR) conditions for mutational analyses

| Mutations | Primers | PCR conditions |
|-----------|---------|----------------|
| KRAS codon 12–13 | F: 5'-GGCTCGCTGAAAAATGACTGAA | 100 at 95°C, 40 cycles of 15" at 95°C, 30" at 64°C, 40" at 72°C and 5" at 72°C |
| | R: 5'-[Btn]-TTGGCTCGCAAATGATTCTGA | |
| | Seq: 5'-TATAACCTTGTGGTATGTTG | |
| KRAS codon 61 | F: 5'-CAGACTGTTGTCTCCCTCTCA | 100 at 95°C, 40 cycles of 15" at 95°C, 30" at 66°C, 30" at 72°C and 5" at 72°C |
| | R: 5'-[Btn]TCTATGGTCTGCTCTCTGATTG | |
| | Seq: 5'-ATATTCTGACACAGCAG | |
| KRAS codon 146 | F: 5'-TGGCAGACGTTTGGAAAGATAATTG | 100 at 95°C, 40 cycles of 15" at 95°C, 30" at 66°C, 30" at 72°C and 5" at 72°C |
| | R: 5'-[Btn]-ATTAGAAGCGATGCTCCCTCAAG | |
| | Seq: 5'-ATATTCTTATTTAGTGAAT | |
| BRAF codon 600 | F: 5'-ATCGCCTGCTCTGATTGAA | 100 at 95°C, 40 cycles of 15" at 95°C, 30" at 54°C, 40" at 72°C and 5" at 72°C |
| | R: 5'-[Btn]-GCATCTCAAGGCCCAA | |
| | Seq: 5'-GGTGATTTTGGTCTAGCTAC | |

Abbreviations: [Btn] = biotinylated; Seq = sequencing primer.
RESULTS

A total of 138 patients with mCRC, who had received cetuximab and irinotecan in four Italian Medical Oncology Units, have been screened for KRAS codons 12 and 13 mutations.

Clinical and pathological characteristics of the overall population and of the study population are summarised in Table 2.

Eighty-seven (63%, 52 men and 35 women) of 138 patients had KRAS codons 12 and 13 wild-type disease and entered the study population. Median age was 66 (range: 41 – 79). Of these 87, 44 (51%), 39 (44%) and 4 (5%) patients had an ECOG PS of 0, 1 and 2, respectively.

All patients received cetuximab plus irinotecan according to the schedule commonly used in clinical practice: cetuximab 250 mg/sqm i.v., day 1 weekly (loading dose: 400 mg/sqm i.v., day 1 in the first cycle) or 500 mg/sqm i.v., day 1 every 2 weeks and irinotecan 180 mg/mq i.v., day 1 every 2 weeks.

In the study population, according to RECIST criteria, 1 CR and 23 PRs were reported, for an overall response rate (RR) of 28%. Of the patients, 35 (40%) achieved SD and 28 (32%) experienced PD.

At the time of the analysis, 81 (93%) patients underwent disease progression and 63 (72%) died. Median PFS and median OS were 4.1 and 9.7 months, respectively.

Mutational status of KRAS codons 61 and 146

The mutational analyses of codons 61 and 146 were successfully performed in 76 out of 87 cases. KRAS was mutated in codon 61 or 146 in 7 (8%) and 1 (1%) cases, respectively. None of the 8 patients bearing KRAS 61 or 146 mutated disease responded to the treatment, whereas 22 (32%) of 68 patients with wild-type disease achieved response ($P$ = 0.096; Table 3B). KRAS 61 and 146 mutations were associated with significantly shorter PFS (median...
PFS: 3.8 vs 5.1 months in KRAS 61 and 146 wild type; HR: 0.46 (0.11–0.88), P = 0.028; Figure 1A), whereas no significant differences were detected in OS (median OS: 9.7 vs 14.7 months in KRAS 61 and 146 wild-type; HR: 0.69 (0.24–1.75), P = 0.390; Figure 1B).

**Mutational status of BRAF codon 600**

Among the 87 patients of the study population, BRAF was mutated in 13 cases (15%). KRAS codons 61, 146 and BRAF V600E mutations were mutually exclusive. 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; Table 3C). BRAF mutation was associated with a trend towards shorter PFS (median PFS: 2.6 vs 4.4 months in BRAF wild-type; HR: 0.59 (0.26–1.2), P = 0.307; Figure 1C) and with significantly shorter OS (median OS: 4.1 vs 13.9 months in BRAF wild-type; HR 0.51 (0.18–0.95), P = 0.037; Figure 1D).

**Combined analysis of KRAS codons 61 and 146 and BRAF codon 600 mutational status**

Among 74 patients with BRAF wild-type disease, KRAS codons 61 and 146 mutational status was successfully assessed in 68 cases. Eight (11%) tumours were KRAS 61 or 146 mutated. None of patients with KRAS 61 or 146 mutated tumours responded, in comparison with 22 (37%) of 60 KRAS 61 and 146 wild-type patients (P = 0.047; Table 4A). Also in BRAF wild-type subpopulation, KRAS codons 61 and 146 mutations were associated with shorter PFS (median PFS: 3.8 vs 5.3 months in KRAS 61 and 146 wild-type; HR: 0.45 (0.10–0.85), P = 0.023; Figure 2A), whereas no significant differences were reported in terms of OS (median OS: 9.7 vs 14.8 months in KRAS 61 and 146 wild-type; HR: 0.32 (0.21–1.66), P = 0.320; Figure 2B).

In the subgroup of 68 patients with KRAS 61 and 146 wild-type tumours, BRAF was mutated in 8 cases. None of BRAF-mutated patients obtained a response, whereas 22 (37%) out of 60 patients with BRAF wild-type disease responded (P = 0.047; Table 4B). BRAF mutation was associated with a trend towards shorter PFS (median PFS: 3.9 vs 5.3 months in BRAF wild-type; HR: 0.69 (0.26–1.52), P = 0.306; Figure 3A) and OS (median OS: 9.0 vs 14.8 months in BRAF wild-type; HR: 0.63 (0.20–1.60), P = 0.279; Figure 3B).
Among 87 patients of the study population, none of 21 patients with tumours presenting whatever mutation responded to the treatment, in comparison with 22 (37%) of 60 patients with KRAS 61 and 146 and BRAF wild-type disease ($P = 0.0005$; Table 5).

Wild-type patients reported significantly longer PFS (median PFS: 5.3 vs 3.3 months; HR: 0.51 (0.22–0.77), $P = 0.006$; Figure 4A) and OS (median OS: 14.8 vs 9.7 months; HR: 0.54 (0.24–0.92), $P = 0.027$; Figure 4B).

**DISCUSSION**

The molecular test for *KRAS* mutations has been introduced in the routine clinical practice of the oncologists facing mCRC (Allegra et al, 2009). Since the first report of *KRAS* predictive value for resistance to cetuximab (Lièvre et al, 2006), investigators looked at most of the frequent mutations (ie, those affecting codons 12 and 13). Therefore, also the most reliable demonstrations of
KRAS mutations as determinants of resistance to anti-EGFR moAbs, that is, those deriving from *post hoc* analyses of randomised studies (Karapetis et al, 2008; Amado et al, 2008; Van Cutsem et al, 2009), investigated only codons 12 and 13 genetic variants. Even if quite specific for nonresponsiveness, KRAS codon 12 and 13 analysis suffers from low sensitivity. In fact around 35% of wild-type patients experience rapid disease progression.

This background led to the search for alternative predictive factors, such as EGFR ligands expression (Khambata-Ford et al, 2007) or polymorphisms (Garm Spindler et al, 2009), alterations in other EGFR signalling pathways (ie, PTEN/PI3K/AKT; Loupakis et al, 2009) or in downstream effectors of KRAS (ie, BRAF). In particular, Di Nicolantonio et al (2008) recently found that none of 11 BRAF-mutated patients, among 79 KRAS codon 12 and 13 wild-type, responded to anti-EGFR moAbs. BRAF mutation also predicted for an unfavourable outcome in terms of both PFS and OS. From a methodological standpoint mutational analyses are quite more appealing with respect to other techniques such as immunohistochemistry or gene expression profiling. In fact, the determination of mutational status is easily reproducible, qualitative, less expensive and does not require fresh tumour tissue sampling. Taking into account these considerations and the growing knowledge on minor oncogenic KRAS mutations in codons 61 and 146 (mutually exclusive with those in codons 12 and 13), we conducted this retrospective study to verify whether the combined analyses of such rare KRAS mutations and BRAF codon 600 variants are related to resistance to cetuximab plus irinotecan.

In the present analysis, 13, 7 and 1 patient among 87 patients with KRAS codon 12 and 13 wild-type disease had tumour bearing BRAF V600E, KRAS codon 61 and KRAS codon 146 mutation, respectively. None of the KRAS codon 12 or 13 wild-type patients bearing an alteration on KRAS codon 61, 146 or BRAF codon 600 responded to treatment. Moreover patients with mutated tumours had a significantly worse outcome both in terms of PFS and OS. Such data indicate that, even if much more rare than codon 12 or 13 mutations, codon 61 and 146 as well as BRAF mutations also seem to predict resistance to cetuximab. As these rare KRAS mutations and BRAF mutations are mutually exclusive with the others, it seems reasonable to test for their presence only in patients with KRAS codon 12 and 13 wild-type tumours.

Considering our data, it seems that among those subjects that are expected not to have codon 12 or 13 mutations (around 60% of mCRC patients), testing for BRAF and rare KRAS mutations would exclude around 25% of patients, with obvious saving of economic resources and sparing unnecessary toxicities.

The quest for the most sensible and specific tools for selecting patients who are more likely to benefit from anti-EGFR inhibitors is an issue acquiring a great relevance for two main reasons. On one hand, in addition to the present indication for the use of anti-EGFR moAbs, the results from a recent phase III randomised trial (Van Cutsem et al, 2009) led to the approval of cetuximab for KRAS codon 12 and 13 wild-type patients also in the first-line setting. On the other hand, it has been proven that in such setting the choice of using an anti-EGFR moAb precludes the possibility of coadministering the antiangiogenic antibody bevacizumab, even in KRAS codon 12 and 13 wild-type patients (Tol et al, 2009; Hecht et al, 2009). Moreover, it should be noted that the choice of the best upfront treatment for each patient is not only to offer the best palliation, but it may even influence the possibility for cure (Adam et al, 2009). In this regard it should be considered that there are different ongoing randomised studies evaluating the impact of moAbs in the adjuvant setting, where the clinical impact of reliable predictors of outcome will be even greater.

In conclusion, our results suggest that KRAS testing power for predicting resistance to anti-EGFR might be improved by including codon 61 and 146 mutational analysis. This finding may have rapid and important implications for routine clinical practice. Moreover, this study confirms the recent finding that indicates BRAF V600E mutation as a promising additional marker for resistance. Such preliminary and retrospective results should be verified on samples from patients enrolled in randomised studies of anti-EGFR moAbs vs best supportive care.

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