Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial

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Background: Evidence suggests that metastatic colorectal carcinoma (mCRC) has a high level of intratumor heterogeneity. We carried out a quantitative assessment of tumor heterogeneity for KRAS, NRAS, BRAF and PIK3CA mutations, in order to assess potential clinical implications.

Patients and methods: Tumor samples (n = 182) from the CAPRI-GOIM trial of first-line cetuximab + FOLFIRI in KRAS exon-2 wild-type mCRC patients were assessed by next-generation sequencing that allows quantitative assessment of mutant genes. Mutant allelic frequency was normalized for the neoplastic cell content and, assuming that somatic mutations usually affect one allele, the Heterogeneity Score (HS) was calculated by multiplying by 2 the frequency of mutant alleles in neoplastic cells. Therefore, HS virtually corresponds to the fraction of neoplastic cells carrying a specific mutation.

Results: The KRAS HS ranged between 12 and 260 with mean value of 87.1 and median value of 84.4, suggesting that in most CRC, the majority of neoplastic cells carry mutant KRAS. Similar findings were observed for NRAS (HS range 35.5–146.7; mean 102.8; median 117.1). In contrast, in BRAF (HS range 17.1–120; mean 54.8; median 54.3) and PIK3CA (HS range 14.3–120; mean 59.5; median 47.3) mutant cases, only a fraction of neoplastic cells seem to carry the mutant allele. The response rate was 70% in KRAS mutant patients with an HS <33 (low KRAS; n = 10) and 45.7% in KRAS HS >33 patients (high KRAS; n = 35); median progression-free survival were 7.97 and 8.37 months, respectively. Low-KRAS tumors had a higher frequency of additional mutations in PIK3CA when compared with high-KRAS (6/10 versus 8/35).

Conclusions: KRAS and NRAS mutations are usually present in the majority of neoplastic cells, whereas BRAF and PIK3CA mutations often affect a limited fraction of transformed cells. Resistance to cetuximab in low-KRAS patients might be driven by the complex mutational profile rather than KRAS mutation load.

Key words: colorectal cancer, mutations, cetuximab, next-generation sequencing, tumor heterogeneity
and 15%–20% of mCRC, respectively [6]. The role of these molecular alterations in the resistance to EGFR monoclonal antibodies is still debated. However, they identify subgroups of patients that might benefit of treatment with drugs capable to interfere with either the RAS/RAF/MEK/ERK or PI3K/AKT pathways [7]. Screening of CRC with high throughput technologies revealed molecular alterations in several different pathways, which might drive resistance to anti-EGFR agents and offer at the same time potential for therapeutic intervention [8].

In addition to the above-described intertumor heterogeneity, CRC has also an important level of intratumor heterogeneity. Different studies have shown that every CRC carries several different molecular alterations [8]. These findings have been recently confirmed by our group by analyzing with a next-generation sequencing (NGS)-based technique tumor samples from the ‘Cetuximab after progression in KRAS wild-type colorectal cancer patients’ (CAPRI)–Gruppo Oncologico dell’Italia Meridionale (GOIM) clinical trial [9]. NGS analysis revealed that several tumor samples had co-existing mutations in different genes. For example, 30/45 cases with mutated KRAS had concomitant mutations in other genes, with samples showing up to five different mutations.

Intriguingly, we noted that, in some cases, the frequency of mutant alleles did not correlate with the fraction of neoplastic cells. In addition, in tumors with multiple mutations, the frequency of mutant alleles was often different among the affected genes, thus suggesting a further level of intratumor heterogeneity. In this respect, here we report a quantitative assessment of tumor heterogeneity for KRAS, NRAS, BRAF and PIK3CA mutations and discuss the potential clinical implications of these findings.

patients and methods

study design and patient population

CAPRI is a nonprofit academic, open-label, multicenter study carried out by the GOIM cooperative group (Eudract number: 2009-014041-81). Patients with KRAS exon 2 wild-type mCRC were treated in first line with FOLFIRI plus cetuximab. After progression, patients were randomized (1:1) to receive every two weeks FOLFOX alone or FOLFOX plus cetuximab as second-line therapy. The second-line treatment part of the study is currently ongoing. The protocol was approved in each participating center by the local independent Ethics Committee. The trial was carried out in accordance with the Declaration of Helsinki. All patients provided both written and oral informed consent.

multiple gene mutation analysis by next-generation sequencing

The fraction of neoplastic cells was assessed by two experienced pathologists (FT and GB). Tumor samples (N.182) were analyzed with the Ion AmpliSeq™ Colon and Lung Cancer Panel (Life Technologies) using Ion Torrent semiconductor sequencing, as previously described [9]. KRAS and BRAF V600E mutations were confirmed by Sanger sequencing and/or real-time PCR.

heterogeneity score

Mutant allelic frequency was normalized for the neoplastic cell content, in order to calculate the frequency of mutant alleles in neoplastic cells. Assuming that somatic mutations usually affect one allele, the HS was calculated by multiplying by 2 the frequency of mutant alleles in neoplastic cells. Therefore, HS virtually corresponds to the fraction of neoplastic cells carrying a specific mutation. In particular, HS = 100 suggests that all neoplastic cells carry the mutation; HS <100 indicates that only a fraction of neoplastic cells is mutant; HS >100 implies copy-number variation, either gain of the mutant allele or loss of the wild-type allele.

results

molecular profiling of mCRC with NGS

The molecular profiling with NGS of 182 tumor samples from the CAPRI trial has been previously reported [9]. In particular, 45 cases (24.7%) had mutations in KRAS with one case carrying two different mutations; 24 (13.2%) in PIK3CA with two tumors having two mutations; 15 (8.2%) in BRAF; and 13 (7.1%) in NRAS (supplementary Table S1, available at Annals of Oncology online). Overall 78/182 tumors had KRAS, NRAS, PIK3CA and/or BRAF mutations. KRAS and NRAS mutations were mutually exclusive, whereas PIK3CA and BRAF mutations were detected in both RAS wild-type and mutant tumors. In particular, 13/26 PIK3CA mutations and 4/15 BRAF variants were identified in RAS mutant tumors. Of note, only 1/10 BRAF V600E mutation was found in a KRAS mutant tumor.

heterogeneity within KRAS, NRAS, PIK3CA and BRAF mutant tumors

The mutant allelic frequency and the fraction of neoplastic cells significantly varied among tumors carrying mutations in KRAS, NRAS, PIK3CA or BRAF (supplementary Table S2, available at Annals of Oncology online). In order to more adequately represent tumor heterogeneity, the HS was calculated for KRAS, NRAS, PIK3CA and BRAF mutant tumors.

The HS values showed a wide range suggesting that heterogeneous content of mutant alleles occurred for all investigated genes (Figure 1 and Table 1). However, the pattern of mutant allele content was clearly different among the four genes. Although the KRAS HS ranged between 12 and 260, 27/45 (60%) tumors showed a HS ≥70 with a HS mean value of 87.1 and median value of 84.4. Similar findings were observed for NRAS, with 10/13 (77%) cases having a HS ≥70, an HS mean of 102.8 and a median of 117.1. In contrast, in BRAF mutant tumors, the HS range was 17.1–120 with a mean of 54.8 and a median of 54.3 and only 4/15 (26.7%) cases had HS ≥70. Interestingly, we found five non-V600E mutations in our cohort of samples, and all these mutant cases showed a low HS (<50). Mean and median HS for BRAF V600E mutant cases were 67.5 and 62.1, respectively. Finally, 8/24 (33.3%) PIK3CA mutant cases showed an HS ≥70. The PIK3CA HS range was 14.3–120, with a mean value of 59.5 and a median of 47.3.

clinical activity of FOLFIRI plus cetuximab according to KRAS HS value

It has long been debated whether it is possible to identify within KRAS mutant tumors a threshold to separate tumors resistant to EGFR monoclonal antibodies from those that might be responsive to these agents. Therefore, we assessed whether the HS value could be useful for this purpose. We arbitrarily set the threshold at 33, reasoning that tumors containing less than...
one-third of KRAS mutant cells might benefit of anti-EGFR therapies. In addition, the 10 KRAS mutant tumors below this threshold had a KRAS allele frequency ranging between 3% and 10%. Therefore, these cases are unlikely to be detected by routine diagnostic methods based on Sanger sequencing.

Among the 10 patients carrying KRAS mutant tumors with HS <33, 7 responded to FOLFIRI plus cetuximab therapy (Table 2). In contrast, patients with a KRAS mutant tumor with HS >33 showed a response rate in line with the activity of FOLFIRI alone (45.7%), as expected in RAS mutant tumors. However, the median progression-free survival (mPFS) was quite similar for the high-KRAS and low-KRAS groups (7.97 versus 8.37 months). The small size of the sample analyzed does not allow any statistical consideration.

These data might suggest that a low content of KRAS mutant alleles is sufficient to produce resistance to EGFR monoclonal antibodies. However, 7/10 low-KRAS tumors had additional mutations in PIK3CA, TP53, BRAF, ERBB2, FGFR3 and/or FBXW7 (supplementary Table S3, available at Annals of Oncology online). Additional mutations were also found in 23/35 (65.7%) tumors with KRAS HS >33, although the mutational landscape was different between low- and high-KRAS

| Table 1. Heterogeneity Score (HS) among different mutant tumor samples |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| No. of cases               | HS Range                    | Mean (95% CI)               | Median (95% CI)              |
| KRAS                       | 45a                         | 12–260                      | 87.1 (84.1–90.1)             | 84.4 (80.6–88.1)             |
| NRAS                       | 13                          | 35.5–146.7                  | 102.8 (96.8–108.8)           | 117.1 (109.5–124.6)          |
| BRAF                       | 15                          | 17.1–120                    | 54.8 (49.3–60.3)             | 54.3 (47.4–61.2)             |
| PIK3CA                     | 24b                         | 14.3–120                    | 59.5 (55.3–63.7)             | 47.3 (42–52.6)               |

1 One case had two KRAS mutations.
2 Two cases had two different PIK3CA mutations.

**Figure 1.** Heterogeneity Score (HS) values of KRAS, NRAS, BRAF and PIK3CA mutations. Section symbol: non-V600E BRAF mutations; asterisk: exon 20 PIK3CA mutations. One case had two exon 2 KRAS mutations and two cases had both exon 9 and 20 PIK3CA mutations; the HS was calculated using the sum of the allelic frequency of both mutations.

| Table 2. KRAS Heterogeneity Score (HS) and efficacy of treatment in the CAPRI trial |
|-----------------------------|-----------------------------|-----------------------------|
| No. Responses ORR (%) (95% CI) Median PFS (months) |
| HS <33                      | 10 3 SD 70 (40–89) 7.97 (6.03–n.a.) |
| 6 PR                        | 1 CR                        |
| HS >33                      | 35 4 PD 45.7 (30–62) 8.37 (5.27–11.8) |
| 15 SD                       | 14 PR                       |
| 2 CR                        |                             |

SD, stable disease; PR, partial response; CR, complete response; ORR, overall response rate.
tumors. In particular, the frequency of PIK3CA mutations was higher in low-KRAS mutant tumors when compared with high-KRAS: 6/10 in low-KRAS tumors versus 8/35 in high-KRAS mutant cases ($P = 0.046$, $\chi^2$ test). The four BRAF mutations concomitant with KRAS mutations were equally distributed between the high- and low-KRAS mutant groups, with two mutations per group ($P = 0.16$, $\chi^2$ test). However, the only BRAF V600E occurred in the low-KRAS group. An example of complex genotype in tumors with low-KRAS mutation content is shown in supplementary Figure S1, available at *Annals of Oncology* online, in which the two tumors from patients with shorter PFS are represented. However, we acknowledge that our analysis does not allow to assess whether mutations are in the same cells or in different cells.

discussion

Here we show for the first time a quantitative estimation of neoplastic cells carrying KRAS, NRAS, BRAF and/or PIK3CA mutations in CRC within a clinical trial. Several studies have addressed the intratumor heterogeneity of KRAS and, at lesser extent, BRAF and PIK3CA mutations in retrospective series of CRC. Heterogeneous distribution of KRAS mutations within primary CRC or between primary tumors and lymph node or distant metastases has been shown in some studies [10–12], but these findings could not be confirmed by others [13, 14]. However, these studies employed qualitative methods for KRAS testing that only allowed to assess the presence or the absence of the mutation.

In order to represent tumor heterogeneity, we coupled quantitative assessment of mutant allele frequency by NGS with normalization for the neoplastic cell content. Although this approach suffers of the approximate estimation of neoplastic cell content, our findings suggest that intratumor heterogeneity does occur in KRAS, NRAS, BRAF and PIK3CA mutant CRC. Indeed, in many tumors only a fraction of neoplastic cells was found to carry the mutant allele, thus confirming that CRC is often formed of clones with different mutational profile. These data are in agreement with previous studies, including the recent ‘Big Bang’ model by which private alterations that give rise to intratumor heterogeneity would be generated early after the transition to advanced tumor and will be ‘pervasive’ in the final neoplasm [15].

Significant differences were found among the four genes that we investigated. The majority of KRAS and NRAS mutant CRC were found to carry the mutant allele in a high fraction of neoplastic cells. In contrast, most of BRAF and PIK3CA mutant tumors showed the mutant allele in a limited fraction of transformed cells. These data suggest that RAS mutations are likely to occur at early stage of CRC tumorigenesis. Interestingly, we found a KRAS mutant tumor with HS 260 that is likely due to an allelic imbalance. Analysis of raw data did not show evidence of gene amplification, and this might indicate deletion of the wild-type allele. BRAF and PIK3CA mutations might occur in later phases of tumor progression in most cases, and cooperate with other molecular alterations to sustain colon cancer progression. Wider molecular characterization of BRAF and PIK3CA mutant tumors are needed to address this issue.

The finding that tumors are highly heterogeneous with respect to a specific mutation might have important implications for therapeutic approaches. We expect that tumors that carry a specific molecular alteration only in a fraction of neoplastic cells might show a low level of sensitivity to targeted agents. Interestingly, BRAF V600E mutant CRC are resistant to treatment with BRAF inhibitors [16], and a mechanism of resistance involving the activation of the EGFR pathway might be involved in this phenomenon [17]. However, we found that only 4/10 BRAF V600E mutant CRC had $\geq$70% of mutant cells, and 2/10 had $<50%$ mutant cells. These findings might at least in part explain the relative resistance of BRAF mutant mCRC to treatment with a single inhibitor directed against the BRAF pathway. Interestingly, 5/15 BRAF mutations were non-V600E. BRAF mutations outside of the V600E codon have been found in different tumor types (lung carcinoma, thyroid cancer etc.) and some have been reported to be oncogenic. Their potential role in colorectal tumorigenesis and drug resistance/sensitivity deserves additional investigation.

The fraction of neoplastic cells carrying a specific molecular alteration could also correlate with the level of resistance to anti-EGFR agents. In this respect, it is not possible to drive firm conclusions from our findings in the KRAS mutant subgroup because of the small sample size. Nevertheless, our data showed a peculiar trend: patients with low-KRAS mutation content responded to EGFR-based therapy, but their PFS was similar to patients with high-KRAS tumors. These data are in line with our current knowledge of resistance to targeted agents: the presence of a low fraction of cells carrying a resistance mutation may not prevent the response to a specific drug, but the duration of the response will be shorter because the resistant clone will expand rapidly and will cause the recurrence of the disease [18]. Our data are also in agreement with a number of previous publications showing that even low levels of KRAS mutations produce resistance to EGFR targeting agents [19, 20]. Quantitative assessment of BRAF and PIK3CA mutations might also allow to better define the role of these molecular alterations in the resistance to anti-EGFR drugs.

We identified in several low-KRAS tumors additional mutations that might play a role in resistance to EGFR targeting agents, such as BRAF and PIK3CA mutations. These findings raise the question of whether resistance to EGFR targeting agents is driven in these patients by the low-level KRAS mutant, by the mutations in PIK3CA, in BRAF or even other genes not included in our panel, or by a cooperative effect of these pathways, which is likely to be the case. Our data also suggest that the classification of tumors in RAS, BRAF or PIK3CA mutant is an attempt to represent a complex phenomenon in a too simplistic, and have revealed the existence of a subgroup of CRC with a ‘mixed’ genotype characterized by different potentially driver mutations. In this regard, quantitative assessment of mutational load might allow identifying the priority target for therapeutic intervention in tumors with a complex mutational profile. However, the complexity revealed by our study suggests that, for many tumors, combinations of targets-based agents will likely to be necessary to control tumor growth.

In conclusion, our attempt of a tri-dimensional representation of the mutational landscape of CRC, although limited to a relatively small number of genes, has revealed an high level of heterogeneity that is likely to affect response to target-based agents. We believe that assessment of such complexity is mandatory for the further development of personalized medicine in mCRC.
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disclosure

The authors have declared no conflicts of interest.

references


appendix

The following GOIM-CAPRI investigators participated to this study and are coauthors of the article: