RAS-expanded Mutations and HER2 Expression in Metastatic Colorectal Cancer: A New Step of Precision Medicine

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Abstract: Cetuximab and panitumumab monoclonal antibodies are a milestone in the history of treatment of metastatic colorectal cancer (mCRC) and point toward future directions for personalized treatment. Recent studies have shown that broader RAS testing is needed to select patients for targeted therapy. The objectives of our study were to identify the prevalence of RAS mutations and evaluate human epidermal growth factor receptor 2 (HER2) expression in KRAS exon 2 wild-type (WT) mCRC patients, correlating the findings with objective response rate, progression-free survival, and overall survival. In total, 29 mCRC patients undergoing treatment with cetuximab therapy were enrolled in this study. By pyrosequencing, mutations were found in 17% of nonresponder patients, in KRAS codon 146 and NRAS codon 12. HER2 positivity was limited to only 1 responder carcinoma specimen. There was no correlation between RAS mutation, HER2/neu expression, and clinicopathologic findings. We highlighted significantly the differences between objective response rate and RAS gene status. The overall survival and progression-free survival of RAS WT patients were higher compared with those with RAS-mutated disease. Clinical response to cetuximab therapy is impaired in the presence of RAS-expanded mutations. In fact, our finding of 5 mutations in RAS-expanded genes allowed us to understand the resistance to cetuximab in 33% of KRAS WT exon 2 nonresponder patients. HER2 does not seem to be a potential biomarker for cetuximab-targeted therapy. These analyses suggest that the assessment of other biomarkers is needed to determine the best treatment for patients with mCRC, to maximize benefit and minimize harm.

Key Words: cetuximab, RAS, HER2, target therapy, colorectal cancer

Metastatic colorectal cancer (mCRC) is a heterogeneous disease that develops through multistep and complex processes of genetic changes driving the transformation from normal epithelium to invasive cancer. A better understanding of the biology of colorectal cancer (CRC) and the identification of the epidermal growth factor receptor (EGFR) pathway, as a crucial mechanism in carcinogenesis, has made it possible to assign biological therapies according to the specific mutational status of each patient. Cetuximab and panitumumab are important treatment options in patients with CRC, being monoclonal antibodies (mAbs) that target the extracellular domain of EGFR. mAbs keep EGFR in an inactive state by binding to it, and occluding the ligand-binding site of EGFR when the ligand is unbound (thus acting as competitive antagonists). This results in an inhibition of the intracellular signaling pathway of EGFR (RAS/RAF/MAPK and PI3K/PTEN/AKT), which is involved in several cellular activities, including cell proliferation, motility, invasion, and survival.

Amado et al3 reported the first analysis of a randomized clinical trial showing that the efficacy of panitumumab was limited to patients with wild-type (WT) KRAS exon 2 (codons 12 and 13) mCRC. Thus, mutant KRAS tumor status was shown to be a negative predictive marker for mCRC. KRAS proteins are small guanine-nucleotide binding proteins that transduce the signal from ligand-bound EGFR to the nucleus. KRAS mutations significantly impair the GTPase activity of KRAS proteins, leading to a constitutive, growth factor receptor–dependent activation of downstream signaling.2 Recent studies, in particular the retrospective analysis of the PRIME trial,4 have suggested that mutations in KRAS outside exon 2 (KRAS exons 3/4 or NRAS exons 2/3/4), referred to as all RAS, predict lack of response to anti-EGFR therapy. Recent analyses have reinforced these findings,5–8 and treatment guidelines now (since 2013) recommend that RAS WT tumor status should be confirmed in all mCRC patients before initiating treatment with these agents. Mutations of KRAS are the major negative predictors of the efficacy of anti-EGFR mAbs. They occur in 30% to 40% of mCRC, predominantly in codon 12 (~70% to 80%) and codon 13 (~15% to 20%) of exon 2. In contrast, patients with “all RAS” WT tumors significantly benefited from the treatment.
BRAF, a member of the RAF family, is a protein kinase encoded by the BRAF gene, which plays an important role as an intermediary in the RAS/RAF signaling cascade.\(^9\)

BRAF and RAS mutations are mutually exclusive, and clinical data suggest that the BRAF V600E mutation is a poor prognostic marker in terms of survival.\(^10\)\(^,\)\(^11\)

Human epidermal growth factor receptor 2 (HER2) is a receptor belonging to the ErbB family whose activation does not require the presence of a ligand.\(^12\)\(^,\)\(^13\) Indeed, it depends on the heterodimerization of HER2 with other similar receptors of the family.\(^12\)\(^-\)\(^15\) Overexpression/amplification of HER2 is an established therapeutic target in breast and gastric cancer.\(^14\)\(^,\)\(^15\) The role of HER2 in CRC is less clear.\(^16\)\(^-\)\(^18\) Some studies have shown that HER2 gene amplification was significantly related to resistance to cetuximab or panitumumab and was associated with a significantly worse progression-free survival (PFS) and a trend toward a worse overall survival (OS).\(^19\)\(^)\(^,\)\(^20\)

In the present study we estimated the prevalence of RAS mutations (KRAS exons 3/4 or NRAS exons 2/3/4) in KRAS exon 2 WT mCRC patients, correlating the findings with objective response rate (ORR), PFS, and OS. Moreover, because of the high percentage of resistance to therapy, in the same CRC patients an additional predictive marker for cetuximab target therapy was taken into account, namely HER2.

**MATERIALS AND METHODS**

**Patient Population**

From March 2006 to September 2013, 1032 CRC patients underwent resection at our Scientific Institute for Digestive Diseases, of whom 350 had mCRCs. KRAS (codons 12 and 13) mutational analysis of metastatic cases was performed and highlighted 210 WT and 140 mutated CRC specimens. In total, 29 of the 210 WT colorectal carcinoma patients chose to undergo treatment with an anti-EGFR antibody (cetuximab in irinotecan combination therapy) at the Medical Oncology Service of our Institution; these are the patients enrolled in this study. No patient received neoadjuvant chemotherapy. Responders (n = 14) were defined as those patients who achieved a complete response and/or partial response; nonresponders (n = 15) were those with stable or progressive disease (PD). PFS was calculated from the start of cetuximab administration until PD or death, whereas OS was defined as the time from the start of cetuximab treatment until death or until the last follow-up. ORR to first cetuximab-based therapy was determined according to the Response Evaluation Criteria in Solid Tumors (RECIST). The clinical-pathologic characteristics of the patients in the “responder” and “nonresponder” groups are reported in Table 1. Mutation analysis was performed with the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method\(^21\) on 29 formalin-fixed paraffin-embedded CRC specimens. The mean age in our patient cohort was 63.4 years, and the median follow-up time was 31 months (range, 4 to 59 mo).

| Patients (n = 29) | Responder (n = 14) | Nonresponder (n = 15) | P |
|------------------|-------------------|-----------------------|---|
| Age (M ± SD) (y) | 64.14 ± 9.3       | 62.33 ± 11.8          | NS |
| Sex [n (%)]      |                   |                       |    |
| Male             | 13 (92.9)         | 10 (66.7)             | NS |
| Female           | 1 (7.1)           | 5 (33.3)              |    |
| Tumor site [n (%)] |                 |                       |    |
| Rectum           | 5 (35.7)          | 6 (40.0)              | NS |
| Left colon       | 7 (50.0)          | 7 (46.7)              |    |
| Right colon      | 2 (14.3)          | 2 (13.3)              |    |
| Histology [n (%)] |                 |                       |    |
| Well differentiated | 0                | 2 (13.3)              | NS |
| Moderately        | 10 (71.4)         | 6 (40.0)              |    |
| Poorly differentiated | 3 (21.4)       | 5 (33.4)              |    |
| Mucinous         | 1 (7.2)           | 2 (13.3)              |    |

NS indicates not significant.

Among the 29 patients, there were 6 (21%) women and 23 (79%) men. Eleven tumors were located in the rectum (38%), 14 in the left-sided colon (48%), and 4 in the right-sided colon (14%). At histology, 2 tumors were well differentiated (7%), 16 were moderately differentiated (55%), 8 were poorly differentiated (28%) adenocarcinomas, and 3 were mucinous carcinomas (10%). The study was performed according to the Declaration of Helsinki and was approved by the local committee for medical research ethics. All subjects gave oral and written consent.

**HER2 Immunohistochemistry (IHC)**

IHC was carried out in an automated autostainer (Dako, Denmark) as previously described.\(^15\) Briefly, 4-µm formalin-fixed paraffin-embedded sections were dewaxed and incubated in 10 mM/L citrate buffer (pH 6) at 98°C for 40 minutes. Following incubation with the primary rabbit antibody to the intracellular domain of the HER2 protein (A0485, Dako), a visualization reagent (DAKO REAL Envision) was applied. Negative controls were created by omission of the primary antibody and replacement with phosphate buffered saline. Known positive tissues were used as the HER2-positive control. The 4-tiered scoring system suggested by Hofmann et al\(^22\) was used for the HER2-staining evaluation.

**HER2 Chromogenic In Situ Hybridization (Dual Color-CISH)**

HER2 CISH staining was performed according to the manufacturer’s instructions (Dako, Denmark). In short, specimens were subjected to heat pretreatment (98°C for 15 min) and pepsin digestion at 37°C to prepare the tissue for probe hybridization. Denaturation for 5 minutes at 82°C and overnight hybridization at 45°C were performed simultaneously for the HER2/Texas Red–labeled DNA probe and the CEN-17/FITC-labeled PNA probe using a Hybridizer (Dako, Denmark). Specimens were subjected to stringent washing at 65°C for 10 minutes.
before transfer to a CISH wash stringent buffer. The signals from the fluorescent probes were converted to chromogenic signals in an IHC staining reaction performed on an automated platform (Autostainer, Dako Denmark). The immunohistochemical staining included blocking of endogenous peroxidase activity, incubation with horse-radish peroxidase–conjugated anti-FITC and alkaline phosphatase–conjugated anti-Texas Red antibodies, and development of chromogenic signals using red and blue chromogens. The slides were dried at 37°C and mounted in a permanent mounting medium. HER2 CISH–stained slides were interpreted using a bright field microscope with 40 × and 60 × lenses. The HER2/CEN-17 ratio was calculated by counting 20 nuclei from the invasive tumor area. On the basis of the ratio, the specimens were assigned to the amplified (HER2/CEN-17 > 2.0) or nonamplified (HER2/CEN-17 < 2.0) categories. Normal cells within the specimen served as internal control for staining success. Normal cells should exhibit the expected ratio for normal diploidy with a one-to-one relationship of red and blue signals.

**KRAS, NRAS, and BRAF Mutation Analysis**

The blocks with the highest proportion of tumor cells over stroma, inflammation, necrosis, mucinous, normal, or adenomatous colonic tissue were selected by a pathologist on hematoxylin and eosin-stained slides, macroscopically circled, and scalpel-dissected.

Depending on the size of the tissue sample, 2 to 5 sections (10-μm thick) of FFPE tissue were used for DNA extraction using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden). The protocol was performed according to the manufacturer’s instructions. DNA quality and concentration (μg/mL) were assessed according to the A260/280 absorbance ratio in an ultraviolet spectrophotometer (Jenway-Genova, Italy).

**DNA Amplification and Pyrosequencing**

As a first step we reevaluated the status of KRAS codons 12, 13 and BRAF codon 600 with a more sensitive technique (pyrosequencing) than the previous RFLP method. Pyrosequencing is a real-time, rapid, and alternative method to Sanger sequencing that can provide quantitative information with limit of detection of 5%. Pyrosequencing determines the exact sequence and provides the same accuracy as Sanger sequencing, but it is less time-consuming when running multiple samples. The same tissue blocks from all 29 patients were used to reextract fresh DNA.

The mutation status of KRAS (codons 12, 13, 59, 61, 117, 146), NRAS (codons 12, 13, 59, 61, 117, 146), and BRAF (codons 464, 466, 469, 600) was determined by pyrosequencing on the Qiagen PyroMark Q24 device according to the CE-IVD-marked therascreen RAS Pyro Kit Handbook (Qiagen, Version 1, July 2011). Briefly, 2×50 ng of genomic DNA combined with codon-specific primers were used for the initial 25 μL PCR reaction volume. After PCR, 10 μL of the amplicons was immobilized on streptavidin-coated beads and denatured to produce single-stranded products. Single-stranded DNA was prepared and the corresponding sequencing primers were allowed to anneal to the DNA. Sequence analysis was performed using PyroMark Q24 software in the AQ (allele quantification) analysis mode.

The quality thresholds for the mutational analysis were a required peak height of 30 relative units for “passed” quality and 10 relative units for “check” quality. Samples with an initial “check” status, or with an indicated mutation signal of 2% to 5%, were subjected to a second round of analysis performed in duplicate. In addition, samples that failed the initial analysis were subjected to a second round of analysis. A negative control (without DNA) and a WT control were run with each series of samples.

**Statistical Analysis**

The variables measured in the study were investigated for association using the Fisher exact test or the χ² test as appropriate. PFS was measured for each patient from the day of treatment to the first event of PD. Survival curves were estimated using the Kaplan-Meier method, and differences among them were evaluated by the log-rank Mantel Cox test. A value <0.05 was considered significant. All statistical analyses were performed using the SPSS pack version.

**RESULTS**

In total, 29 mCRC patients undergoing treatment with cetuximab were enrolled in this study. Regarding KRAS codons 12, 13 and BRAF codon 600, the results obtained by pyrosequencing and RFLP showed a perfect match between the 2 methods. By RAS extension analysis, among the 29 mCRC specimens with WT in codons 12 and 13 of KRAS, we detected mutated tumors in 5 cases (17%). The details are as follows: 3 patients (10%) had a KRAS codon 146 mutation (A146V) and 2 patients (7%) had mutations in NRAS codon 12 (G12S 3.5% and G12V 3.5%) (Fig. 1).

There were no significant differences when correlating ORR to the other clinical-pathologic characteristics listed in Table 1. By Fisher exact 2-sided test we correlated ORR with the RAS gene status and found statistically significant differences in terms of response versus nonresponse between RAS-mutated and all RAS WT tumors (P = 0.04) (Table 2).

Regarding PFS, all patients with all RAS WT tumors had a longer median PFS compared with those with RAS-mutated disease (12 vs. 8 mo, P = 0.05 by log-rank test) (Fig. 2). Moreover, all patients with all RAS WT tumors had a longer median OS compared with those with RAS-mutated tumors (median OS, 31 vs. 17 mo, P = 0.58) (Fig. 3).

HER2 was evaluated using IHC and CISH in 29 tissues from mCRC patients. HER2 IHC expression revealed that 28 samples (97%) showed little or no HER2 expression (score 0 to 1+), whereas only 1 case (3%) had a moderate (score 2+) HER2 IHC expression (Fig. 4).
HER2 amplification, evaluated by CISH, was observed only in the same sample with the 2+ IHC score. The amplification was present in >10% of neoplastic cells (Fig. 5). Therefore, the only HER2-positive case belonged to a 54-year-old man with rectal adenocarcinoma (G2 histologic grading) RAS/BRAF WT, who was classified as a responder patient.

**DISCUSSION**

Since 2013, RAS mutations have been considered the most important predictive biomarker of resistance to anti-EGFR therapy in mCRC, and the only marker approved for clinical use. RAS gene testing has therefore become an important part of the workup of CRC patients. Patients enrolled in this study were eligible for anti-EGFR therapy because they had WT in codons 12 and 13 of the KRAS; 48% of them benefited from the therapy.

The purpose of this study was to determine the incidence of mutations in RAS extension codons (KRAS codons 59, 61, 117, 146 and NRAS codons 12, 13, 59, 61, 117, 146) and consequently determine whether the mutation status of RAS modified the effect of cetuximab on ORR, OS, and PFS.

By RAS extension pyrosequencing analysis, we identified a further 5 (33.3%) RAS mutations in nonresponder patients (Table 2). The overall incidence of RAS extension mutations was similar to that described in other studies, but unlike what was reported in the literature (a higher incidence of mutations in codon 61 than in codon 12 of NRAS), we found mutations in NRAS codon 12, not in codon 61. This finding can be due to geographic factors and sex, which are the only variables associated with overall and individual exon RAS mutation prevalence, although the reasons for variations in RAS mutations between countries are still unclear. Little is known regarding the impact of NRAS mutation in CRC. Some studies have shown that NRAS mutations seem to arise at a later stage in the development of malignancy, unlike KRAS mutations, which arise early.

All 5 patients (3 women and 2 men) with additional mutations were nonresponders, confirming the negative effect of RAS mutations on the outcome after cetuximab treatment. In fact, this result explains the resistance to therapy in 33% of nonresponder patients (Table 2) allowing the finding of a statistically significant difference in terms of response versus nonresponse between RAS-mutated and purely all RAS WT tumor patients ($P = 0.04$) (Table 2).

Significantly shorter median PFS (6 vs. 12 mo, $P = 0.05$) was found for patients with mutant RAS tumors compared with RAS WT patients (Fig. 2). Although

![FIGURE 1](https://example.com/figure1.png)

**FIGURE 1.** The distribution of KRAS and NRAS mutations in 5 nonresponder patients.

![FIGURE 2](https://example.com/figure2.png)

**FIGURE 2.** Kaplan-Meier survival analysis for progression-free survival time of patients with and without RAS mutation.

![FIGURE 3](https://example.com/figure3.png)

**FIGURE 3.** Kaplan-Meier survival analysis for overall survival time according to RAS status.

| Gene | Codon | Analyte | Mutation Detected |
|------|-------|---------|-------------------|
| KRAS | 146   | A146V   | 437 C>T, GCA>GTA |
| NRAS | 12    | G12S    | 34G>A, GGT>AGT   |
| NRAS | 12    | G12D    | 35G>T, GGT>GTG   |

**TABLE 2. RAS Status in Responder Versus Nonresponder Patients**

|          | RAS Mut | RAS WT |
|----------|---------|--------|
| Responder [n (%)] | 0       | 14 (100) |
| Nonresponder [n (%)] | 5 (33.3) | 10 (66.7) |

The Fisher exact test $P = 0.04$.

Mut indicates mutation; WT, wild-type.
not statistically significant, because of the small number of patients, the median OS was higher in patients without RAS mutations compared with that in RAS-mutated patients (31 vs. 17 mo, \( P = 0.58 \)) (Fig. 3). However, despite the 5 mutated cases, there were still a considerable number of nonresponder patients who had WT RAS genes (66.7%). One reason for this could be that RAS has no direct interaction with the anti-EGFR antibody at the antigen site. It would therefore be prudent to investigate the expression of other members of the HER family, which have been shown to activate the downstream pathways, through heterodimerization and cross-talk, and drive the tumorigenesis of CRC in these patients.

Herein, we observed HER2 positivity in only 1 sample (3.4%) of CRC patients belonging to the responder RAS WT group. The low percentage of HER2 positivity in our study is in agreement with figures reported by other authors.\(^{17,29}\) The HER2 amplification did not show a diffuse pattern but was present in a minority of cells. This would explain, as suggested by Martin et al.\(^{30}\) the sensitivity of these patients to cetuximab treatment. These authors\(^{30}\) evaluated HER2 gene status in 170 KRAS WT (codon 12, 13, 61) mCRC patients treated with cetuximab or panitumumab, and identified 3 profiles: (a) patients with no or slight HER2 amplification; (b) patients with HER2 amplification in minor clones or with increased HER2 gene copy number due to polysomy (HER2-CNG); and (c) patients with HER2 amplification in all cells. The worst outcome was seen in the group of patients with amplification in all cells, whereas intermediate outcomes were seen in patients with no amplification. Interestingly, the best outcomes were seen in the group of patients with amplification in minor clones or polysomy. The authors explain these different results by supposing that tumors in the group with HER2 amplification in a minority of the cells or with HER2 polysomy may have a different pathogenesis linked to a general chromosome instability. In the group with HER2 amplification in all cells, however, HER2 activation can bypass the blockade of EGFR mediated by panitumumab and cetuximab, inducing a strong resistance to these mAbs. The lack of efficacy of anti-EGFR treatment for the patients with no amplification of HER2 has been related to karyotypic heterogeneity.\(^{30}\) However, unlike our study, they did not assess all RAS status. Although the small number of samples does not allow any assessment of the significance of HER2 as a therapeutic target predictive of the efficacy of cetuximab therapy, it can be stated that HER2 alone is not responsible for the high percentage of CRC cases resistant to cetuximab therapy. We confirm that a relevant proportion of patients (17%) considered KRAS exon 2 WT have an additional mutation in the RAS pathway. Thus, a RAS mutation is predictive of nonresponse to anti-EGFR therapies but alone it is not a sufficient basis on which to decide who should not receive such therapies, because \( \sim 50\% \) (40% to 60%) of RAS WT CRC patients show a poor response to anti-EGFR-based treatment. This fact leads us to suspect that there must be other molecular determinants of response to anti-EGFR therapies that have not yet been identified, which would allow us to refrain from futile treatments and related toxicities. Finally, RAS testing is still an ongoing field, and in the following years research efforts for RAS WT patients will be focused on the evaluation of additional markers of cetuximab or panitumumab primary resistance.

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