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Clinical Cancer Research

Increased Detection Sensitivity for *KRAS* Mutations Enhances the Prediction of Anti-EGFR Monoclonal Antibody Resistance in Metastatic Colorectal Cancer

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Abstract

Purpose: *KRAS* mutations represent the main cause of resistance to anti-epidermal growth factor receptor (EGFR) monoclonal antibodies (MoAbs) in metastatic colorectal cancer (mCRC). We evaluated whether highly sensitive methods for *KRAS* investigation improve the accuracy of predictions of anti-EGFR MoAbs efficacy.

Experimental Design: We retrospectively evaluated objective tumor responses in mCRC patients treated with cetuximab or panitumumab. *KRAS* codons 12 and 13 were examined by direct sequencing, MALDI-TOF MS, mutant-enriched PCR, and engineered mutant-enriched PCR, which have a sensitivity of 20%, 10%, 0.1%, and 0.1%, respectively. In addition, we analyzed *KRAS* codon 61, *BRAF*, and *PIK3CA* by direct sequencing and PTEN expression by immunohistochemistry.

Results: In total, 111 patients were considered. Direct sequencing revealed mutations in codons 12 and 13 of *KRAS* in 43/111 patients (39%) and *BRAF* mutations in 9/111 (8%), with almost all of these occurring in nonresponder patients. Using highly sensitive methods, we identified up to 13 additional *KRAS* mutations compared with direct sequencing, all occurring in nonresponders. By analyzing *PIK3CA* and PTEN, we found that of these 13 patients, 7 did not show any additional alteration in the PI3K pathway.

Conclusions: The application of highly sensitive methods for the detection of *KRAS* mutations significantly improves the identification of mCRC patients resistant to anti-EGFR MoAbs. *Clin Cancer Res; 17(14); 4901–14. ©2011 AACR.*

Introduction

Cetuximab and panitumumab are monoclonal antibodies (MoAbs) that inhibit the activation of the epidermal growth factor receptor (EGFR) and its downstream pathways, namely the RAS-RAF-MAPK and the PI3K-PTEN-AKT axes (1). As the response rate to anti-EGFR MoAbs varies from 10% to 20% in patients with metastatic colorectal cancer (mCRC; ref. 2), several studies have been performed to identify markers that can predict the efficacy of these agents.

It is widely accepted that a lack of response to anti-EGFR MoAbs occurs in the presence of oncogenic *KRAS* mutations (3–11). This finding led the European Medicines Agency and, subsequently, the Food and Drug Administration to limit the use of cetuximab and panitumumab only to patients with wild-type *KRAS* tumors (12, 13).

In addition, the presence of oncogenic deregulation of EGFR and other members of its downstream signaling pathways, such as *BRAF*, *PIK3CA*, and PTEN, might influence the response to cetuximab and panitumumab and could, therefore, help to identify nonresponder (NR) patients (4, 14–19). However, the evaluation of these additional molecular markers does not seem to be sufficient to fully predict the response to EGFR-targeted agents (20, 21). This lack could be explained by inappropriate or

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Translational Relevance

The analysis of *KRAS* mutations has been approved by the Food and Drug Administration and EMEA as a diagnostic tool to select metastatic colorectal cancer patients eligible to be treated with cetuximab or panitumumab. However, standardized techniques for *KRAS* analysis are lacking, and the test sensitivity required to identify nonresponder (NR) patients has yet to be determined. Here, we show that by analyzing *KRAS* with highly sensitive methods (matrix-assisted laser desorption/ionization—time-of-flight [MALDI-TOF] MS (mass spectrometry), mutant-enriched PCR, and engineered mutant-enriched PCR (eME-PCR), characterized by 10%, 0.1%, and 0.1% sensitivity, respectively), we were able to increase the rate of NR identification from 45% (detected by direct sequencing of *KRAS*) to 62%. Our data point to the importance of increasing the sensitivity of methods to detect *KRAS* mutations for enhancing predictions of resistance to cetuximab or panitumumab in mCRC. This result shows an urgent need for the establishment of widely accepted *KRAS* testing guidelines.

nonstandardized methodologies and by the limited sensitivity of current sequencing methods for detecting DNA point mutations (22).

Despite a general consensus favoring the introduction of *KRAS* testing in clinical practice as a powerful means to select patients before drug administration, validated and standardized techniques for *KRAS* analysis are still lacking (23–25). Currently, the most widely applied method for assessing *KRAS* gene status is direct sequencing, which has a relatively low sensitivity because mutant alleles must be present in at least 20% of cells to be reproducibly detected (25).

More sensitive methods are available for *KRAS* analysis. Some are laboratory-made techniques, such as mutant enriched-PCR (ME-PCR), and others are CE-marked commercial tests for diagnostic use, such as matrix-assisted laser desorption/ionization—time-of-flight (MALDI-TOF) technology or ARMS. Several studies have compared different methodologies of *KRAS* analysis but without showing a correlation with the clinical response to anti-EGFR agents in mCRC patients (26, 27).

In this study, highly sensitive methods for the detection of *KRAS* mutations (ME-PCR and MALDI-TOF MS) have been evaluated to identify patients that are unlikely to benefit from anti-EGFR MoAbs. In addition to *KRAS* mutations in codons 12 and 13, we analyzed the more infrequent mutations occurring at codon 61 by direct sequencing. Finally, to better understand the impact of *KRAS* tests on predicting the efficacy of EGFR targeted drugs, we also analyzed the mutational status of *BRAF* and *PIK3CA* and protein expression of PTEN in the same cohort.

Materials and Methods

Patient population and treatment regimens

We retrospectively analyzed 111 patients with histologically confirmed mCRC collected from 1996 to 2009. All tumors were colorectal adenocarcinomas, diagnosed at the Institute of Pathology of Locarno (Switzerland; $n = 52$), the Civic Hospital of Legnano (Italy; $n = 22$), the University Foundation of Chieti (Italy; $n = 30$), and at the University School of Medicine of Novara (Italy; $n = 7$). Patients gave informed consent and were treated with cetuximab- or panitumumab-based regimens at the referred institutions. All patients had EGFR expression in at least 1% of neoplastic cells, as detected by immunohistochemical studies.

In chemotherapy-refractory patients, cetuximab was administered as a single agent or in combination with chemotherapy-based regimens (irinotecan or oxaliplatin in 95 and 7 patients, respectively) given at the same dose and schedule used at progression. Therefore, the patients included in this study were selected based primarily on evidence that the treatment outcome could only be attributed to the administration of either cetuximab or panitumumab.

With the exception of 3 patients who received cetuximab as frontline monotherapy, the others had failed at least 1 prior chemotherapy regimen.

Cetuximab, as monotherapy or in combination with another chemotherapeutic regimen, was administered at a loading dose of 400 mg/m^2 over 2 hours, followed weekly by 250 mg/m^2 over 1 hour. Panitumumab (6 mg/kg) was given i.v. every 2 weeks until progression.

Clinical evaluation and tumor response criteria

Treatment was continued until progressive disease (PD) or toxicity occurred, according to the standard criteria (28). The clinical response was assessed every 6 to 8 weeks by radiologic examination. The Response Evaluation Criteria in Solid Tumors (RECIST; 28) were adopted for clinic evaluation, and the objective tumor response was classified as partial response (PR), stable disease (SD), or PD. Patients with SD or PD were defined as NR (28). Two independent oncologists and radiologists verified the clinical response for all patients in a blinded manner.

Molecular analyses

Formalin-fixed paraffin-embedded tumor blocks were reviewed for quality and tumor content. A single representative tumor block for each case, containing at least 70% neoplastic cells, was selected for immunohistochemical, cytogenetic, and molecular analyses. Tumor macrodissection was performed in tumor blocks containing less than 70% of neoplastic cells (to reduce the presence of non-neoplastic tissues).

Mutational analysis of *KRAS*, *BRAF*, and *PIK3CA* by direct sequencing

Genomic DNA was extracted using the QIAamp Mini kit (Qiagen) according to the manufacturer's instructions.

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Table 1. Primers used for the PCR reactions

Gene	Exon	Method	Forward primer	Reverse primer
KRAS	2	DS	TGGTGGAGTATTTGATAGTGTA	CATGAAAATGGTCAGAGAA
KRAS	3	DS	GGTGCACTGTAATAATCCAGAC	TGATTTAGTATTATTTATGGC
KRAS cod 12	2	ME-PCR (first PCR)	ACTGAATATAAACTTGTGGTAG-TTGGACCT	ACTCATGAAAATGGTCAGAG-AAACCTTTAT
KRAS cod 12	2	ME-PCR (second PCR)	ACTGAATATAAACTTGTGGTAG-TTGGACCT	TCAAAGAATGGTCCTGGACC
KRAS cod 13	2	ME-PCR (first PCR)	ACTGAATATAAACTTGTGGTAGTT-GGCCCTGGT	ACTCATGAAAATGGTCAGA-GAAACCTTTAT
KRAS cod 13	2	ME-PCR (second PCR)	ACTGAATATAAACTTGTGGTAG-TTGGCCCTGGT	TCAAAGAATGGTCCTGCACC
BRAF	15	DS	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA
PIK3CA	9	DS	GGGAAAAATATGACAAAGAAAGC	CTGAGATCAGCCAAATTCAGTT
PIK3CA	20	DS	CTCAATGATGCTTGGCTCTG	TGGAATCCAGAGTGAGCTTTC

Abbreviations: cod, codon; DS, direct sequencing; ME-PCR, mutant-enriched PCR.

Mutational analyses by direct sequencing were performed at the Institute of Pathology in Locarno (Switzerland).

Mutations in *KRAS* (exons 2–3, containing hotspot codons 12, 13, and 61), *BRAF* (exon 15, containing codon 600), and *PIK3CA* (exons 9 and 20, containing codons 542, 545, and 1047) were detected by direct sequencing (sensitivity of about 20%) of genomic DNA as previously described (17,19,29). The list of primers used for the mutational analyses is reported in Table 1. Direct sequencing has a sensitivity of approximately 20%.

PTEN expression

PTEN protein expression status was analyzed using immunohistochemistry (IHC) on 3 μm tissue sections as reported previously (4).

Mutational analysis of *KRAS* by highly sensitive techniques (MALDI-TOF MS, ME-PCR, eME-PCR)

Mutations at codons 12 and 13 of the *KRAS* gene were investigated using MALDI-TOF MS (specific only for G12V, G12D, G12A, G12C, G12R, G12S, G12F, G13D, G13V; sensitivity of about 10%), ME-PCR and eME-PCR (both with a sensitivity of 0.1%).

MALDI-TOF MS technology

The analysis of *KRAS* using MALDI-TOF was performed by staff from the Institute for Cancer Research and Treatment (IRCC), Candiolo, Italy, at Sequenom GmbH, Hamburg, Germany. This advanced technology for high-throughput mutational analysis of tumor samples is based on a combination of PCR and MALDI-TOF MS, using the MassARRAY System (Sequenom GmbH). *KRAS* exon 2 was amplified by PCR using OncoCarta PCR primers (Sequenom). The thermocycling conditions were as follows: 94°C for 2 minutes; 45 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 5 minutes. The primary PCR reaction mix was treated with shrimp alkaline phosphatase to deactivate unincorporated dNTPs, and a single-base primer extension step was performed. This method is based on the annealing of a primer adjacent to the mutation site and extension with 1 mass-modified dNTP. The reaction was cycled at 94°C for 30 seconds; followed by 40 cycles of 94°C for 5 seconds, 52°C for 5 seconds, and 80°C for 5 seconds; and a final extension at 72°C for 3 minutes. CLEAN resin (Sequenom) was added to the mixture to remove extraneous salts that could interfere with the MALDI-TOF analysis. Finally, the extended primers were dispensed onto a 384-well SpectroCHIP II array (Sequenom), using a MassARRAY Nanodispenser (Samsung), and analyzed by a MALDI-TOF mass spectrometer. The data were processed using a MassARRAY Typer 4.0 Analyzer (Sequenom). MALDI-TOF MS has a sensitivity of approximately 10%.

ME-PCR

KRAS analyses by ME-PCR were performed at the Institute of Pathology in Locarno (Switzerland). ME-PCR consists of 2 amplification steps (semi-nested PCR), in which artificial restriction sites are introduced into the wild-type amplicon using mismatched primers (30). The restriction site, BstNI for codon 12 or BglI for codon 13, introduced during the first PCR step, is positioned immediately next to the *KRAS* codon being analyzed to distinguish between the wild-type and mutant sequences. Wild-type amplicons were then digested by BstNI or BglI, whereas mutant products were enriched for a second round of amplifications. The ME-PCR and digestion conditions have been reported previously (30). The list of primers used for the mutational analyses is shown in Table 1. All samples were subjected to automated sequencing by an ABI PRISM 3130 (Applied Biosystems) using reverse primers. All mutated cases were confirmed twice with independent PCR reactions. ME-PCR has a sensitivity of up to 0.1% (30).

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eME-PCR

KRAS analyses by eME-PCR were performed at the Clinical Research Center in Chieti (Italy). An eME-PCR technique was recently developed to further increase the detection sensitivity of *KRAS* mutations at codons 12 and 13. The method has previously been described in detail and used for the detection of *KRAS* mutations in lung adenocarcinomas (31). This technique has been engineered to obtain the highest sensitivity and specificity through the optimal selection of reagents and several modifications to the original protocol. eME-PCR has a sensitivity of approximately 0.1%.

Statistical considerations

The objective tumor response was used as the endpoint of our study. A 2-tailed Fisher's exact test was used to calculate the *P* values for the association between gene alterations and the clinical response to anti-EGFR MoAbs. The level of significance was set at $P = 0.05$. The progression-free survival (PFS) and overall survival (OS) analysis were performed according to the Kaplan–Meier method, and survival curves were compared using the log-rank test. OS was defined as the time from the start of treatment with cetuximab or panitumumab until the last follow-up, whereas PFS was calculated from the start of treatment with cetuximab or panitumumab until the first documented tumor progression or death. The data were analyzed using the SAS System V9.1 (SAS Institute Inc.).

Results

Patient characteristics

This study analyzed 111 patients, including 27 patients already considered in our previous work (4). The patients' characteristics, treatment regimens, and response by treatment with anti-EGFR MoAbs are summarized in Table 2. After cetuximab- or panitumumab-based therapy, 21 patients (19%) achieved PR.

Mutational profiling of *KRAS* and *BRAF* by direct sequencing

Direct sequencing identified *KRAS* exon 2 mutations in primary tumors in 43 cases (39%). These mutations occurred only in codon 12 in 31 cases (74%) and only in codon 13 in 11 cases (26%); a double-point mutation involving both codons was detected in 1 case (2%). For the analysis of the clinical response, the case with the double mutation was grouped with the codon 12 mutations. Exon 3 of *KRAS* could not be analyzed in 3 cases because of a lack of material. In exon 3, a *KRAS* point mutation was detected in 4/108 evaluable cases (4%). Three cases showed a mutation involving the third nucleotide of codon 61, leading to a Q61H change in 2 cases and a Q61L change in 1 case; the other patient showed a mutation in codon 60 (G60D). *BRAF* mutations were found in 9/111 patients (8%), all represented by V600E substitutions. All *KRAS* mutated cases were *BRAF* wild type and vice-versa, confirming data from the literature (32).

Table 2. Clinico-pathological characteristics

Patient characteristic ($N = 111$)	Number of cases	Percentage (%)
Age		
≤60 y	46	41
>60 y	65	59
Gender		
Male	65	59
Female	46	41
Primary tumor site		
Colon	69	62
Rectum	41	37
Other[a]	1	1
EGFR-targeted therapies		
Cetuximab	6	5
Cetuximab + chemotherapy	102	92
Panitumumab	3	3
No. of previous chemotherapy treatments		
None	3	3
One	18	16
Two	73	66
Three	16	14
Four	1	1
Response to EGFR-targeted therapies		
Partial response	21	19
Nonresponders	90	81

[a]Other: in this case, the primary tumor sites were multiple (colon and rectum).

Mutational profiling of *KRAS* by MALDI-TOF MS technology and ME-PCRs

KRAS analyses by ME-PCR and eME-PCR (hereafter collectively referred to as ME-PCRs) were performed on the entire cohort of mCRC patients. However, it was only possible to analyze *KRAS* gene status by MALDI-TOF MS in 53 patients because of a lack of histological tissue samples from the remaining patients (Table 3). All 3 methodologies showed an increase in sensitivity compared with direct sequencing due to either the application of mass spectrometry (MALDI-TOF MS) or the disruption of wild-type alleles through 2 sequential enzymatic reactions and subsequent re-amplification of mutated alleles (ME-PCRs). ME-PCRs, but not MALDI-TOF MS, are more sensitive than other commercial kits based on ARMS, such as the DxS *KRAS* mutational test, for which the sensitivity is approximately 1%. Moreover, the 3 methods all showed a reproducibility of 100%, as all the mutations found by direct sequencing of codon 12 or 13 of the *KRAS* gene were confirmed by MALDI-TOF MS (in the 53 evaluable patients) and by ME-PCRs (in the entire cohort), and the mutations found by all 3 methodologies were confirmed in 2 independent experiments.

Focusing our attention on the subgroup of patients who had been found to be wild type in exon 2 of *KRAS* by direct

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Table 3. Immunohistochemical and molecular data and clinical response

		Direct sequencing		KRAS exon 2 by the highly sensitive methods			Direct sequencing		IHC
n	Response	KRAS exon 2	KRAS exon 3	MALDI-TOF MS	ME-PCR	eME-PCR	BRAF	PIK3CA	PTEN
1	PD	WT	WT	WT	**G12D**	**G12D**	WT	WT	POS
2	PD	G13D	WT	G13D	G13D	G13D	WT	WT	POS
3	PD	WT	WT	**G12V**	**G12V**	**G12V**	WT	WT	POS
4	PD	WT	WT	WT	WT	WT	WT	WT	NE
5	SD	WT	WT	**G12A**	**G12A**	**G12A**	WT	WT	POS
6	PD	WT	WT	WT	WT	WT	V600E	WT	POS
7	PD	WT	WT	WT	WT	WT	WT	WT	NE
8	SD	G12A	WT	G12A	G12A	G12A	WT	WT	POS
9	PR	WT	WT	WT	WT	WT	WT	WT	POS
10	SD	WT	WT	WT	WT	WT	WT	WT	NEG
11	PD	WT	WT	WT	WT	WT	WT	WT	POS
12	PD	WT	WT	WT	WT	WT	WT	WT	NEG
13	SD	G13D	WT	G13D	G13D	G13D	WT	WT	POS
14	PR	WT	WT	WT	WT	WT	WT	WT	POS
15	PD	G13D	WT	G13D	G13D	G13D	WT	WT	NEG
16	PD	G12A	WT	G12A	G12A	G12A	WT	WT	POS
17	PD	G12A	WT	G12A	G12A	G12A	WT	WT	POS
18	PD	G12D	WT	G12D	G12D	G12D	WT	WT	NEG
19	PD	WT	WT	WT	WT	WT	V600E	WT	NEG
20	PR	WT	WT	WT	WT	WT	WT	WT	POS
21	PR	WT	G60D	WT	WT	WT	WT	WT	POS
22	PR	WT	WT	WT	WT	WT	WT	WT	POS
23	PD	WT	WT	WT	WT	WT	WT	WT	NEG
24	PD	G13D	WT	G13D	G13D	G13D	WT	H1047R	NEG
25	PD	G12D	WT	G12D	G12D	G12D	WT	WT	POS
26	PR	WT	WT	WT	WT	WT	WT	WT	POS
27	PD	WT	WT	WT	WT	WT	V600E	WT	POS
28	PD	G12C	WT	G12C	G12C	G12C	WT	WT	NEG
29	PR	WT	WT	WT	WT	WT	WT	WT	POS
30	PR	WT	WT	WT	WT	WT	WT	WT	POS
31	SD	G12D	WT	G12D	G12D	G12D	WT	WT	POS
32	PD	WT	WT	**G12A**	**G12A**	**G12A**	WT	WT	NEG
33	PD	G12D	WT	G12D	G12D	G12D	WT	WT	POS
34	SD	WT	WT	WT	WT	WT	WT	H1047R	POS
35	PD	WT	Q61H	WT	**G12D**	**G12D**	WT	H1047R	NEG
36	SD	WT	WT	WT	WT	WT	WT	WT	NEG
37	PR	WT	WT	WT	WT	WT	WT	WT	POS
38	SD	WT	WT	**G12V**	**G12V**	**G12V**	WT	WT	NEG
39	SD	WT	WT	WT	WT	WT	WT	WT	POS
40	PD	WT	WT	WT	WT	WT	V600E	E545K	POS
41	PD	WT	WT	WT	WT	**G12D**	WT	WT	POS
42	PR	WT	WT	WT	WT	WT	WT	WT	POS
43	SD	G12S	WT	G12S	G12S	G12S	WT	WT	POS
44	PD	WT	WT	WT	WT	**G12S**	WT	WT	NEG
45	SD	WT	WT	WT	WT	WT	WT	WT	POS
46	SD	WT	WT	WT	**G13C**	**G13C**	WT	WT	NEG
47	PD	G12R+G13D	WT	G12R+G13D	G12R	G12R+G13D	WT	WT	POS
48	PD	G12A	WT	G12A	G12A	G12A	WT	WT	NEG
49	SD	G12A	WT	G12A	G12A	G12A	WT	WT	POS

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Table 3. Immunohistochemical and molecular data and clinical response (Cont'd)

		Direct sequencing		KRAS exon 2 by the highly sensitive methods			Direct sequencing		IHC
n	Response	KRAS exon 2	KRAS exon 3	MALDI-TOF MS	ME-PCR	eME-PCR	BRAF	PIK3CA	PTEN
50	SD	WT	WT	WT	WT	WT	WT	WT	POS
51	PR	WT	WT	WT	WT	WT	WT	WT	NEG
52	PD	WT	WT	WT	WT	WT	WT	WT	NEG
53	SD	G12A	WT	G12A	G12A	G12A	WT	WT	POS
54	PD	G13D	WT	ND	G13D	G13D	WT	E545K	POS
55	PD	WT	WT	ND	WT	WT	WT	WT	POS
56	SD	G12A	WT	ND	G12A	G12A	WT	WT	POS
57	PD	WT	WT	ND	WT	WT	WT	WT	POS
58	SD	G12V	WT	ND	G12V	G12V	WT	WT	NE
59	PR	WT	WT	ND	WT	WT	WT	WT	NEG
60	PD	WT	WT	ND	WT	WT	V600E	WT	POS
61	PD	WT	WT	ND	WT	WT	WT	WT	NEG
62	PD	G13D	WT	ND	G13D	G13D	WT	WT	POS
63	PR	WT	WT	ND	WT	WT	WT	WT	POS
64	PD	WT	WT	ND	WT	WT	WT	WT	POS
65	PR	G13D	WT	ND	G13D	G13D	WT	WT	POS
66	PD	WT	WT	ND	WT	WT	WT	E542K	POS
67	SD	G12V	WT	ND	G12V	G12V	WT	WT	POS
68	PD	WT	WT	ND	**G12D**	WT	WT	WT	POS
69	PD	WT	WT	ND	WT	WT	WT	WT	POS
70	PR	WT	WT	ND	WT	WT	WT	WT	POS
71	SD	G12C	WT	ND	G12C	G12C	WT	WT	POS
72	SD	G12A	WT	ND	G12A	G12A	WT	E545K	POS
73	PD	G12C	WT	ND	G12C	G12C	WT	WT	NEG
74	PD	G12A	WT	ND	G12A	G12A	WT	WT	NEG
75	SD	WT	WT	ND	WT	WT	WT	WT	POS
76	PR	WT	WT	ND	WT	WT	WT	WT	NE
77	PD	G12C	WT	ND	G12C	G12C	WT	WT	NEG
78	PD	G12D	WT	ND	G12D	G12D	WT	WT	POS
79	SD	WT	WT	ND	WT	WT	WT	WT	NE
80	SD	WT	WT	ND	WT	WT	WT	WT	NE
81	PD	WT	WT	ND	WT	WT	V600E	WT	NE
82	PD	WT	NE	ND	WT	WT	WT	NE	NE
83	PD	G12S	NE	ND	G12S	G12S	WT	NE	NE
84	PD	WT	WT	ND	WT	WT	WT	WT	NE
85	PD	WT	Q61L	ND	WT	WT	WT	WT	NEG
86	SD	G12V	WT	ND	G12V	G12V	WT	WT	NE
87	PR	G13D	WT	ND	G13D	G13D	WT	WT	NE
88	PD	G12V	WT	ND	G12V	G12V	WT	WT	NEG
89	PD	WT	WT	ND	WT	WT	WT	WT	NE
90	SD	WT	NE	ND	**G13S**	**G13S**	WT	WT	NE
91	PR	WT	WT	ND	WT	WT	WT	WT	NE
92	SD	G12D	WT	ND	G12D	G12D	WT	WT	NE
93	PD	G12V	WT	ND	G12V	G12V	WT	WT	POS
94	PD	G13D	WT	ND	G13D	G13D	WT	WT	POS
95	SD	WT	WT	ND	WT	WT	V600E	WT	NE
96	SD	G12D	WT	ND	G12D	G12D	WT	E545K	NEG
97	SD	G12V	WT	ND	G12V	G12V	WT	WT	NE

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Table 3. Immunohistochemical and molecular data and clinical response (Cont'd)

		Direct sequencing		KRAS exon 2 by the highly sensitive methods			Direct sequencing		IHC
n	Response	KRAS exon 2	KRAS exon 3	MALDI-TOF MS	ME-PCR	eME-PCR	BRAF	PIK3CA	PTEN
98	PR	WT	WT	ND	WT	WT	WT	WT	NE
99	PD	WT	Q61H	ND	WT	WT	WT	E545G	NE
100	PD	G13D	WT	ND	G13D	G13D	WT	E545K	POS
101	PR	WT	WT	ND	WT	WT	WT	WT	NE
102	PD	G12D	WT	ND	G12D	G12D	WT	E545K	POS
103	PR	WT	WT	ND	WT	WT	WT	WT	POS
104	PD	WT	WT	ND	WT	WT	WT	WT	POS
105	PD	WT	WT	ND	WT	WT	V600E	WT	POS
106	PD	G13D	WT	ND	G13D	G13D	WT	WT	POS
107	PD	G12V	WT	ND	G12V	G12V	WT	WT	POS
108	PD	WT	WT	ND	WT	**G12V**	WT	WT	POS
109	PD	WT	WT	ND	WT	**G12S**	WT	WT	POS
110	PD	WT	WT	ND	WT	WT	V600E	WT	NEG
111	SD	G12A	WT	ND	G12A	G12A	WT	WT	POS

Abbreviations: eME-PCR, engineered mutant-enriched PCR; IHC, immunohistochemistry; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time; ME-PCR, mutant-enriched PCR; ND, not done; NEG, negative expression; NE, not evaluable; PD, progressive disease; POS, positive expression; PR, partial response; SD, stable disease; WT, wild type.
The 13 *KRAS* mutated cases found only by MALDI-TOF MS and/or by ME-PCRs are in bold.

sequencing, we detected additional *KRAS* mutations in 4/36 (11%) analyzable cases (2 G12A and 2 G12V mutations) using the MALDI-TOF MS method. In the entire cohort of 68 patients who showed no mutations in codon 12 or 13 of the *KRAS* gene by direct sequencing, the ME-PCR found the same mutations detected by MALDI-TOF MS and 5 additional mutated cases (3 with G12D, 1 with G13C and 1 with G13S changes). Finally, eME-PCR detected the same mutated cases as ME-PCR, aside from 1 case with a G12D mutation, and 4 additional *KRAS* codon 12 mutations (2 G12S, 1 G12D, and 1 G12V changes) (Table 3, Fig. 1). Overall, in the group for which it was possible to analyze *K-RAS* status by MALDI-TOF, ME-PCR, and eME-PCR, the MALDI-TOF MS technique failed to identify 5/9 mutations detected only by ME-PCRs. All *KRAS* mutations detected by the 3 highly sensitive methodologies were found in *BRAF* wild-type patients, further confirming the mutual exclusivity of mutations in these 2 genes in CRC. In 1 patient (#35, Table 3), a *KRAS* exon 3 mutation was detected in addition to a *KRAS* exon 2 G12D mutation; the latter was found only by ME-PCR.

Overall, through the application of highly sensitive *KRAS* analysis methods, we detected additional *KRAS* alterations in up to 13/68 patients (19%) after direct sequencing had shown them to be wild type in codons 12 and 13 of *KRAS*.

Metastatic lesions (lung and brain in 1 case, and distant extraintestinal metastatic lymph nodes in another) were available in 2 of the 13 patients whose *KRAS* mutations were only detectable with the highly sensitive technologies (patients #1 and #3, respectively, Table 4). The analysis of the metastatic lesions by direct sequencing of *KRAS* was able to reveal the same mutation that was only found in the primary tumors by ME-PCR (Fig. 2).

Mutational profiling of *PIK3CA* by direct sequencing and PTEN protein expression by IHC

Because of a lack of histological tissue samples, cases 2 and 21 were not evaluated for *PIK3CA* and PTEN protein expression, respectively. We detected *PIK3CA* mutations in 11/109 patients (10%) by direct sequencing and loss of PTEN expression in 26 (of 90 evaluable) patients (29%) by IHC. *PIK3CA* mutations were found in exon 9 in 8 cases (73%) with the classical E545K mutation in 6 cases, E545G in 1 case, and E542K in 1 case. Mutations in exon 20 were found in 3 cases (27%), and all were H1047R changes.

Clinical response to anti-EGFR MoAb therapies

The relationship between the *KRAS*, *BRAF*, and *PIK3CA* mutations and PTEN expression with clinical outcome was evaluated in terms of the objective tumor response (Table 5).

With the exception of 2 patients with *KRAS* G13D mutations and 1 patient with the rare *KRAS* G60D mutation in exon 3, the patients with *KRAS*, *BRAF*, or *PIK3CA* mutations, as detected by direct sequencing, did not respond to cetuximab- or panitumumab-based therapy. All but 2 patients showing a loss of PTEN expression by IHC did not respond to anti-EGFR MoAbs. In the entire

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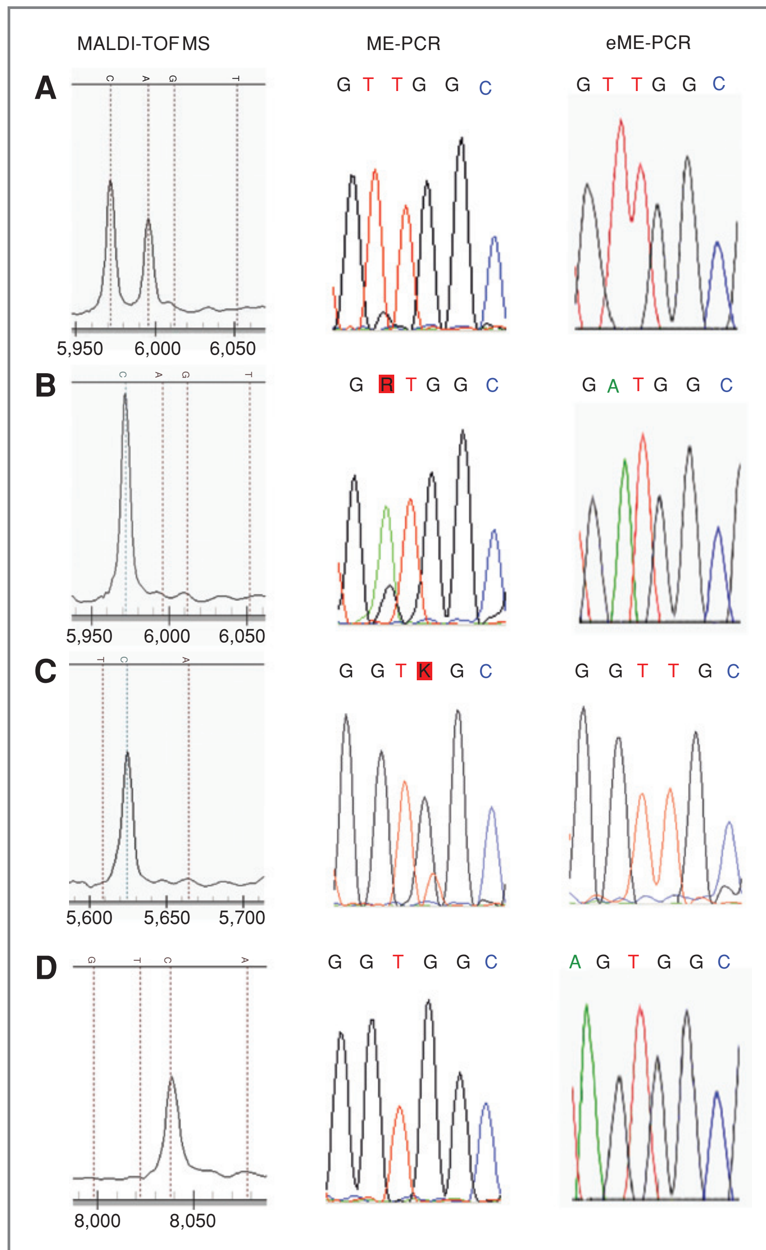

Figure 1. Representative examples of *KRAS* mutations analyzed by MALDI-TOF MS, mutant-enriched PCR (ME-PCR) and eME-PCR found in *KRAS* wild-type cases by direct sequencing. A, a *KRAS* mutation detected by all the 3 high-sensitive methodologies (GGT→GtT, Gly-Val, G12V change, patient #38). B, a *KRAS* mutation detected only by ME-PCR in heterozygosity by ME-PCR and in homozygosity by eME-PCR (GGT→GaT, Gly-Asp, G12D change, patient #1). C, a *KRAS* mutation detected only by ME-PCR, in heterozygosity by ME-PCR and in homozygosity by eME-PCR (GGC-tGC, Gly-Cys G13C change, patient #46). D, a *KRAS* mutation detected only by engineered ME-PCR (homozygous alteration: GGT-aGT, Gly-Ser, G12S change, patient #44).

cohort, only *KRAS* exon 2 status was strongly linked to the response ($P = 0.002$, 2-tailed Fisher's exact test, Table 5). In the group of patients wild type for *KRAS* exon 2, the *KRAS* mutations found by ME-PCR were significantly linked to nonresponse ($P = 0.01$, 2-tailed Fisher's exact test), whereas *BRAF* mutations were borderline linked to anti-EGFR MoAb resistance ($P = 0.053$, 2-tailed Fisher's exact test). *PIK3CA* and PTEN alterations were not associated with response ($P = 0.3$ and 0.1 respectively, 2-tailed Fisher's exact test; Table 5). In the group of *KRAS* wild-type patients, as determined by ME-PCR, *BRAF* mutations were associated with nonresponse ($P = 0.02$, 2-tailed Fisher's exact test), whereas mutations in the other molecular markers were not. Overall, in the subgroup of patients for which all the molecular markers were evaluated (90 cases), alterations in at least 1 molecular marker were found in 61/90 (68%) cases, 57/74 (77%) of those in NR patients.

All cases with *KRAS* mutations detected by MALDI-TOF MS and ME-PCR occurred in NR patients, therefore increasing the rate of identified NR patients from 45% (based on the detection of *KRAS* mutations by direct sequencing alone) to 60% (by adding the analysis of *KRAS* exon 2 by ME-PCR; Fig. 3). Of the 13 patients showing a *KRAS* mutation by ME-PCR, 5 showed a concomitant alteration in *PIK3CA*, *KRAS* codon 61, or PTEN protein expression; 1

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Table 4. Immunohistochemical and molecular data and clinical response in primary tumors and paired metastatic lesions from 2 patients whose *KRAS* mutations were only detected using the highly sensitive methods

			Direct sequencing		KRAS exon 2 by the highly sensitive methods			Direct sequencing		IHC
n	Tissue	Response	KRAS exon 2	KRAS exon 3	MALDI-TOF MS	ME-PCR	eME-PCR	BRAF	PIK3CA	PTEN
1	PT	PD	WT	WT	WT	G12D	G12D	WT	WT	POS
	M lung		G12D	WT	WT	G12D	G12D	WT	WT	POS
	M brain		G12D	WT	WT	G12D	G12D	WT	WT	POS
3	PT	PD	WT	WT	G12V	G12V	G12V	WT	WT	POS
	M LN		G12V	WT	G12V	G12V	G12V	WT	WT	POS

Abbreviations: eME-PCR, engineered mutant-enriched PCR; IHC, immunohistochemistry; LN, lymph node; M, metastasis; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time; ME-PCR, mutant-enriched PCR; NEG, negative expression; NV, not valuable; PD, progressive disease; POS, positive expression; PT, primary tumor; WT, wild type.

was not evaluable for PTEN expression, whereas the 7 remaining cases did not carry any alterations in the pathways downstream of EGFR. Notably, *KRAS* codon 61 mutations were detected in 3 NR patients and occurred concomitantly with H1047R *PIK3CA* mutations, PTEN loss, and mutations in *KRAS* codon 12 that were detected by ME-PCR. Overall, the analysis of the molecular markers by direct sequencing or immunohistochemistry and of *KRAS* mutations by ME-PCR enabled us to identify 87% of the NR patients (Fig. 3).

Kaplan–Meier curves indicated that there were no statistically significant differences in PFS and OS between patients with a *KRAS* wild-type tumor and patients carrying a *KRAS* mutated tumor detected either by direct sequencing or ME-PCR (data not shown).

Discussion

KRAS is the major negative regulator of EGFR-targeted therapies in mCRC patients (3–11). However, no standardized procedures for *KRAS* mutational testing have been proposed and established (23, 24). Indeed, most of the works dealing with the role of *KRAS* in predicting the response to anti-EGFR MoAbs have been performed by means of direct sequencing or allele-specific PCR (25, 33), which have a sensitivity of approximately 10% to 20% for detecting single amino acid substitutions, whereas few studies have used ARMS (1% sensitivity) for *KRAS* analysis in a clinical setting (8, 34). *KRAS* mutational analysis techniques, such as ME-PCR or PCR-RFLP, characterized by increased sensitivity compared with direct sequencing and ARMS, have only been used for detecting micrometastasis in CRC patients (35), either in stool or in serum as a prototype screening (36–38), but have never been used for the assessment of predictive markers in primary tumors. Our study focused on the possibility of improving the detection of *KRAS* mutations in mCRC patients treated with cetuximab or panitumumab using 3 highly sensitive techniques, MALDI-TOF MS, characterized by a sensitivity of 10% (39), and 2 different ME-PCRs techniques, characterized by a sensitivity threshold of 0.1% (40). Increased efficacy of detecting mutated minor clones would lead to more accurate identification of NR patients.

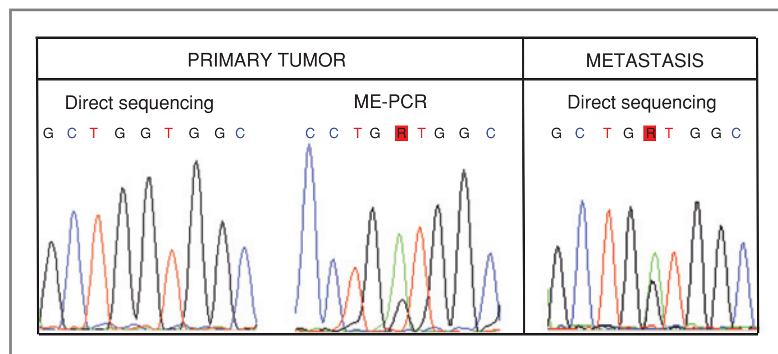

Figure 2. Representative example of the differences in *KRAS* mutational status between a primary tumor and its related metastases in mCRC. The *KRAS* gene was found to be wild type in the primary tumor by direct sequencing and mutated in the related metastatic lesion (GGT-GaT, Gly-Asp, G12D change, patient #1). In the primary tumor, the *KRAS* G12D mutation was detected only by ME-PCR.

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Table 5. Correlation of molecular marker status with the clinical response to anti-EGFR MoAbs

		WT/normal (%)	Mut/loss (%)	*P*
KRAS exon 2	Responders	19 (90)	2 (10)	**0.002**
	Nonresponders	49 (54)	41 (46)	
KRAS exon 2 by the highly sensitive tests (in KRAS wt by DS)	Responders	19 (100)	0 (0)	**0.01**
	Nonresponders	36 (73)	13 (26)	
BRAF exon 15 (in KRAS wt by DS)	Responders	19 (100)	0 (0)	0.053
	Nonresponders	40 (82)	9 (18)	
BRAF exon 15 (in KRAS wt by ME-PCRs)	Responders	19 (100)	0 (0)	**0.02**
	Nonresponders	27 (75)	9 (25)	
PIK3CA exons 9,20 (in KRAS wt by DS)[a]	Responders	19 (100)	0 (0)	0.3
	Nonresponders	43 (90)	5 (10)	
PIK3CA exons 9,20 (in KRAS wt by ME-PCRs)[b]	Responders	19 (100)	0 (0)	0.28
	Nonresponders	31 (88)	4 (12)	
PTEN IHC (in KRAS wt by DS)[a]	Responders	13 (87)	2 (13)	0.1
	Nonresponders	24 (63)	14 (37)	
PTEN IHC (in KRAS wt by ME-PCRs)[b]	Responders	13 (87)	2 (13)	0.17
	Nonresponders	17 (65)	9 (25)	

Abbreviations: DS, direct sequencing; IHC, immunohistochemistry; mut, mutated; wt, wild type; *P* values measured by Fisher's exact test.

[a]*PIK3CA* and PTEN were not evaluable in 1 and 15 *KRAS* wt cases, respectively.

[b]PIK3CA and PTEN were not evaluable in 1 and 14 KRAS wt cases, respectively. The statistically significant differences are shown in bold.

Several steps were undertaken to ensure that optimal procedures were used for mutational testing through direct sequencing. These steps included strict adherence to current recommendations for tissue handling (24) and the involvement of experienced pathologists in selecting representative tissue samples and performing tumor macrodissection. Furthermore, mutational analyses were performed using widely accepted protocols (4, 17), and the laboratory is registered by external quality control audits.

In our cohort, direct sequencing revealed *KRAS* mutations in 43/111 cases (39%), a result in line with the published data on this topic (4, 17, 20). Using more highly sensitive techniques, we found the same alterations detected by direct sequencing, and additional *KRAS* mutations in up to 13 cases were identified, depending on the methodology used, bringing the number of *KRAS* mutations identified to 56/111 cases (50%). The fact that it was possible to perform MALDI-TOF MS analysis only in 50% of cases renders it somewhat difficult to predict the relative contribution of the ME-PCR techniques and MALDI-TOF MS in the entire cohort. However, if we focus on the 53 patients for whom all the analyses were available, MALDI-TOF MS missed 5/9 mutations that were found only by ME-PCRs.

Focusing on works investigating the *KRAS* mutational status of mCRC patients treated with EGFR-targeted therapies, the mutational rate reported in the literature is 32% to 42% when direct sequencing was used and 40% to 45% when ARMS was applied. Therefore, the use of ARMS slightly increases the number of *KRAS* mutations detected. In our cohort, when considering the cases in which it was possible to use direct sequencing and the 3 highly sensitive methods, the *KRAS* mutational status was 32% with direct sequencing (17/53), 40% with MALDI-TOF MS (21/53), and 50% with ME-PCRs (26/53). Therefore, we can postulate that the use of MALDI-TOF MS may lead to the identification of a number of *KRAS* mutations similar to that obtained by ARMS whereas the application of ME-PCRs significantly increases the detected *KRAS* mutational rate.

In addition, when considering *BRAF*, a marker recently linked to MoAb resistance (17, 41), the MAPK pathway was altered in 65/111 patients (58%), thus showing that this axis plays a pivotal role in CRC development. The 3 highly sensitive techniques confirmed all of the mutations in *KRAS* exon 2 found by direct sequencing, indicating that the increase in the sensitivity of our methodologies did not compromise the accuracy of detecting specific *KRAS* mutations.

The data generated by the use of more sensitive techniques are reproducible because all of the mutations found were confirmed at least twice with 2 independent experiments. Moreover, ME-PCR techniques were also used to analyze *KRAS* mutations in plasma samples from CRC patients, and the mutations found corresponded in each case to the same mutation detected in the primary tumor. No mutations were found in patients with *KRAS* wild-type tumors (42). There are some limitations of the high-sensitive techniques we used. ME-PCRs are time-consuming (3 working days) and require sequencing confirmation and

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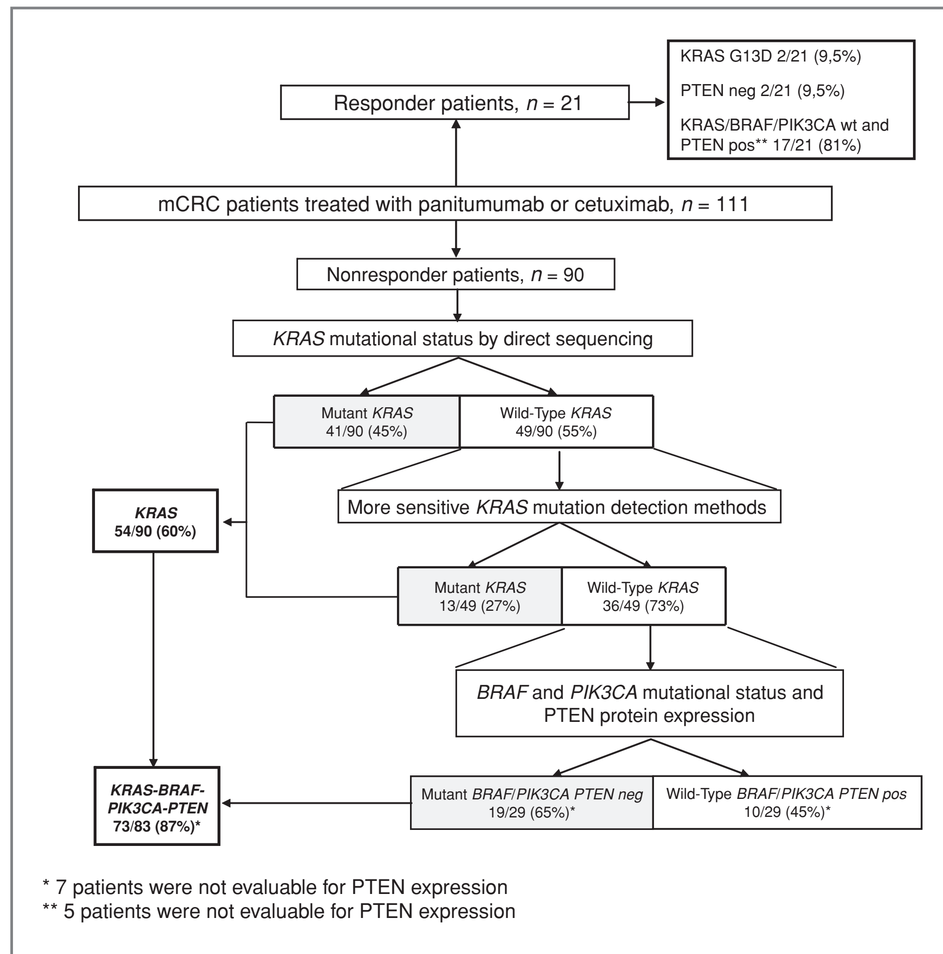

Figure 3. Correlation between clinical data and molecular marker alterations in mCRC patients treated with anti-EGFR MoAbs. *KRAS* mutations detected by direct sequencing identified 45% of nonresponder (NR) patients. More sensitive *KRAS* mutation detection methods increased the identification rate for NR patients to 60%. By adding the evaluation of *BRAF*, *PIK3CA*, and PTEN, we identified up to 87% of NR patients. In the group of responder patients ($n = 21$), 2 showed a *KRAS* mutation (the G13D mutation in both cases), and 2 showed PTEN loss.

considerable manual input to avoid contamination. MALDI-TOF MS is a fast, high-throughput methodology, characterized by lower sensitivity than ME-PCRs and elevated costs. Moreover, it requires 180 ng DNA for the *KRAS* analysis, whereas for ME-PCR, 100 ng is sufficient.

With regard to the response to the anti-EGFR MoAbs, it is important to stress that although the patients included in our cohort are characterized by different chemotherapy background, they represent a homogeneous group in terms of evaluating the cetuximab or panitumumab response, as they experienced resistance to previous chemotherapy regimens.

By correlating the molecular and clinical data, we found that almost all of the *KRAS* exon 2 mutations detected by direct sequencing and all of the *KRAS* exon 2 mutations detected by the highly sensitive techniques occurred in NR patients. Therefore, the use of ME-PCRs increased the rate of identification of NR patients from 45% (detected by direct sequencing only) to 62%. As all *BRAF* mutations were found in NR patients, confirming previous studies (3, 17, 41), by adding the *BRAF* test to the *KRAS* analyses, we identified 63/90 (70%) patients refractory to MoAbs. Our data confirm the results of studies comparing different *KRAS* mutation analysis methods, showing a greater proportion of *KRAS* mutated cases when more highly sensitive methods are used instead of direct sequencing (26, 27). The novelty of this study is that we showed the clinical impact of using a highly sensitive *KRAS* analysis method for patient selection.

Kaplan–Meier analysis showed that patients with *KRAS* mutations, detected by either method, showed similar survival curves in terms of both PFS and OS, with *P*-values not reaching statistical significance. Therefore, our data do not support the prognostic role of *KRAS* in the follow-up of mCRC patients treated with anti-EGFR therapies. However, there are conflicting data in the literature on this topic, so our results are not unexpected (9, 43).

Interestingly, in our cohort, 2 patients carrying both a *KRAS* mutation in codon 13 (the classical G13D change), as identified by direct sequencing, benefited from the treatment. This finding is in line with the literature; a recent review (21) reported that a very small number of patients carrying *KRAS*-mutated tumors responded to either cetuximab or panitumumab. In those patients, codon 13 mutations were predominantly present. A recent study investigating this issue confirmed our results, thus indicating that, in rare instances, *KRAS* codon 13 mutations may not impair the response to cetuximab and panitumumab (44).

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The identification of a greater number of *KRAS*-mutated cases by ME-PCR in primary tumor specimens may be explained by the heterogeneity of the tumors (45–49) and suggests that clones bearing *KRAS* mutations might be undetectable when direct sequencing is used (31). Cells from these clones may display an increased capability to disseminate into peripheral organs where they could predominate (distant metastasis). Our results seem to support this hypothesis; the 2 cases in which *KRAS* mutations were identified only by means of ME-PCR in the primary tumor and for which a metastatic lesion was available showed the same *KRAS* mutation in the metastatic specimens simply by using direct sequencing. The clinical relevance of highly sensitive methodologies is strengthened by the evidence that mutations in *KRAS* gene found only by ME-PCR were never concomitant with *BRAF* mutations, thus confirming the mutual exclusivity between alterations in these 2 genes, even when high-sensitive methodologies of *KRAS* analysis were used.

Recent data have suggested that alterations in the PI3K pathway (e.g., *PIK3CA* gene mutations and the loss of PTEN protein expression) may represent additional negative genetic regulators of EGFR-targeted therapies (4, 17, 19). Therefore, to analyze the clinical significance of *KRAS* mutations that could be detected only by ME-PCRs, we also investigated *PIK3CA* mutations and PTEN protein expression in the same patients. All but 2 patients with PTEN loss, characterized by alterations in the PI3K axis, were NR, thus confirming previous reports (18, 19, 41). In particular, of the 13 cases with a *KRAS* mutation that could be detected only by ME-PCR, 7 did not carry any mutations in the PI3K pathway; therefore, in these cases, the nonresponsiveness is attributable to the presence of the *KRAS* mutation detected by ME-PCR. These results confirm that the use of ME-PCR methods may represent a useful tool for improving the identification of patients unlikely to benefit from MoAb administration. Overall, by examining the PI3K pathway in addition to the *KRAS* and *BRAF* analyses, we were able to identify up to 87% of NR patients.

Because recent reports suggest that rare mutations in *KRAS* exon 3 could be associated with a lack of response to treatment with cetuximab plus irinotecan (41, 50), we extended our analysis of this gene. In the present cohort of patients, we identified 3 mutations in codon 61, all occurring in NR patients (who also showed a *PIK3CA* point mutation or a loss of PTEN expression), and 1 mutation in codon 60, in a patient who responded to therapies. Although they were obtained in a relatively limited number of cases, our results seem to indicate that the examination of *KRAS* exon 3 does not improve the identification of NR patients. These results also suggest that such mutations play a minor role in EGFR-targeted drug efficacy.

Overall, our results suggest that patients might also benefit from the development of sensitive methodologies for the analysis of other predictive molecular markers involved in pathways downstream of EGFR, for example, a ME-PCR technique is also available for *BRAF* (51). However, this assay is not feasible for all mutational analyses because it involves the generation of a restriction site specific for the wild-type allele, which depends on the surrounding sequence. Other more sensitive methods (e.g., allele-specific PCR) may still be developed.

In conclusion, our data point to the usefulness of increasing the sensitivity of methods to detect mutations in *KRAS* for enhancing predictions of resistance to cetuximab or panitumumab in mCRC. There is, therefore, an urgent need for the establishment of widely accepted guidelines for *KRAS* testing, focused on defining the sensitivity threshold that is required the accurate identification of NR patients. We must also emphasize that, as our work is a preliminary and retrospective study, our data will need to be confirmed in larger prospective studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med 2008;358:1160–74.
2. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337–45.
3. Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, Zanon C, Moroni M, Veronese S, et al. Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res 2007;67:2643–8.
4. Frattini M, Saletti P, Romagnani E, Martin V, Molinari F, Ghisletta M, et al. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. Br J Cancer 2007;97:1139–45.
5. Di Fiore F, Blanchard F, Charbonnier F, Le Pessot F, Lamy A, Galais MP, et al. Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. Br J Cancer 2007;96:1166–9.
6. De Roock W, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Personeni N, et al. KRAS wildtype state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. Ann Oncol 2008;19:508–15.

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Published OnlineFirst June 1, 2011; DOI: 10.1158/1078-0432.CCR-10-3137

7. Lièvre A, Bachet JB, Boige V, Cayre A, Le Corre D, Buc E, et al. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol 2008;26:374–9.
8. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008; 26:1626–34.
9. Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.
10. Van Cutsem E, Köhne CH, Hitre E, Zaluski J, Chang Chien CR, Makhson A, et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. N Engl J Med 2009;360:1408–17.
11. Bokemeyer C, Bondarenko I, Makhson A, Hartmann JT, Aparicio J, de Braud F, et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. J Clin Oncol 2009;27:663–71.
12. European Medicine Agency. Available from: http://www.emea.europa.eu.
13. FDA U.S. Food and drug administration: Cetuximab (erbitux) and Panitumumab (vectibux). Available from: http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm172905.htm.
14. Sartore-Bianchi A, Moroni M, Veronese S,, Carnaghi C, Bajetta E, Luppi G, et al. Epidermal growth factor receptor gene copy number and clinical outcome of metastatic colorectal cancer treated with panitumumab. J Clin Oncol 2007;25:3238–45.
15. Cappuzzo F, Finocchiaro G, Rossi E, Jänne PA, Carnaghi C, Calandri C, et al. EGFR FISH assay predicts for response to cetuximab in chemotherapy refractory colorectal cancer patients. Ann Oncol 2007;19:717–23.
16. Jhawer M, Goel S, Wilson AJ, Montagna C, Ling YH, Byun DS, et al. PIK3CA mutation/PTEN expression status predicts response of colon cancer cells to the epidermal growth factor receptor inhibitor cetuximab. Cancer Res 2008;68:1953–61.
17. Di Nicolantonio F, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. J Clin Oncol 2008;26:5705–12.
18. Perrone F, Lampis A, Orsenigo M, Di Bartolomeo M, Gevorgyan A, Losa M, et al. PI3KCA/PTEN deregulation contributes to impaired responses to cetuximab in metastatic colorectal cancer patients. Ann Oncol 2009;20:84–90.
19. Sartore-Bianchi A, Martini M, Molinari F, Veronese S, Nichelatti M, Artale S, et al. PIK3CA mutations in colorectal cancer are associated with clinical resistance to EGFR-targeted monoclonal antibodies. Cancer Res 2009;69:1851–7.
20. Sartore-Bianchi A, Di Nicolantonio F, Nichelatti M, Molinari F, De Dosso S, Saletti P, et al. Multi-determinants analysis of molecular alterations for predicting clinical benefit to EGFR-targeted monoclonal antibodies in colorectal cancer. PLoS One 2009;4:e7287.
21. Bardelli A, Siena S. Molecular mechanisms of resistance to cetuximab and panitumumab in colorectal cancer. J Clin Oncol 2010;28: 1254–61.
22. Marchetti A, Gasparini G. K-ras mutations and cetuximab in colorectal cancer. N Engl J Med 2009;360:833–4.
23. Allegra CJ, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, et al. American Society of Clinical Oncology Provisional Clinical Opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 2009;27:2091–6.
24. Van Krieken JH, Jung A, Kirchner T, Carneiro F, Seruca R, Bosman FT, et al. KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch 2008;453:417–31.
25. Jimeno A, Messersmith WA, Hirsch FR, Franklin WA, Eckhardt SG. KRAS mutations and sensitivity to epidermal growth factor receptor inhibitors in colorectal cancer: practical application of patient selection. J Clin Oncol 2009;27:1130–6.
26. Franklin WA, Haney J, Sugita M, Bemis L, Jimeno A, Messersmith WA. KRAS mutation: comparison of testing methods and tissue sampling techniques in colon cancer. J Mol Diagn 2010;12:43–50.
27. Oliner K, Juan T, Suggs S, Wolf M, Sarosi I, Freeman DJ, et al. A comparability study of 5 commercial KRAS tests. Diagn Pathol 2010;5:23.
28. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000;92:205–16.
29. Frattini M, Balestra D, Suardi S, Oggionni M, Alberici P, Radice P, et al. Different genetic features associated with colon and rectal carcinogenesis. Clin Cancer Res 2004;10:4015–21.
30. Bearzatto A, Conte D, Frattini M, Zaffaroni N, Andriani F, Balestra D, et al. p16(INK4A) Hypermethylation detected by fluorescent methylation-specific PCR in plasmas from non-small cell lung cancer. Clin Cancer Res 2002;8:3782–7.
31. Marchetti A, Milella M, Felicioni L, Cappuzzo F, Irtelli L, Del Grammastro M, et al. Clinical implications of KRAS mutations in lung cancer patients treated with tyrosine kinase inhibitors: an important role for mutations in minor clones. Neoplasia 2009;11:1084–92.
32. Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatchrepair status. Nature 2002;418:934.
33. Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, et al. Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet Oncol 2008;9:962–72.
34. Douillard JY, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol 2010;28:4697–705.
35. Schimanski CC, Linnemann U, Berger MR. Sensitive detection of K-ras mutations augments diagnosis of colorectal cancer metastases in the liver. Cancer Res 1999;59:5169–75.
36. Doolittle BR, Emanuel J, Tuttle C, Costa J. Detection of the mutated K-Ras biomarker in colorectal carcinoma. Exp Mol Pathol 2001;70:289–301.
37. Salbe C, Trevisiol C, Ferruzzi E, Mancuso T, Nascimbeni R, Di Fabio F, et al. Molecular detection of codon 12 K-RAS mutations in circulating DNA from serum of colorectal cancer patients. Int J Biol Markers 2000;15:300–7.
38. Haug U, Hillebrand T, Bendzko P, Löw M, Rothenbacher D, Stegmaier C, et al. Mutant-enriched PCR and allele-specific hybridization reaction to detect K-ras mutations in stool DNA: high prevalence in a large sample of older adults. Clin Chem 2007;53:787–90.
39. Thomas RK, Baker AC, Debiasi RM, Winckler W, Laframboise T, Lin WM, et al. High-throughput oncogene mutation profiling in human cancer. Nat Genet 2007;39:347–51.
40. Nollau P, Wagener C. Methods for detection of point mutations: performance and quality assessment. IFCC Scientific Division, Committee on Molecular Biology Techniques. Clin Chem 1997;43: 1114–28.
41. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, et al. Effects of KRAS, BRAF, NRAS and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol 2010;11:753–62.
42. Frattini M, Gallino G, Signoroni S, Balestra D, Lusa L, Battaglia L, et al. Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. Cancer Lett 2008;263: 170–81.
43. Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter ''RASCAL'' study. J Natl Cancer Inst 1998;90:675–84.
44. De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, et al. Association of KRAS p.G13D mutation with outcome in

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patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. JAMA 2010;304:1812–20.

45. Martin V, Mazzucchelli L, Frattini M. An overview of the EGFR fish challenge in tumor pathology. J Clin Pathol 2009;62:314–24.

46. Giaretti W, Rapallo A, Sciutto A, Macciocu B, Geido E, Hermsen MA. Intratumor heterogeneity of k-ras and p53 mutations among human colorectal adenomas containing early cancer. Anal Cell Pathol 2000;21:49–57.

47. Albanese I, Scibetta AG, Migliavacca M, Russo A, Bazan V, Tomasino RM, et al. Heterogeneity within and between primary colorectal carcinomas and matched metastases as revealed by analysis of Ki-ras and p53 mutations. Biochem Biophys Res Commun 2004; 325:784–91.

48. Losi L, Baisse B, Bouzourene H, Benhattar J. Evolution of intratumoral genetic heterogeneity during colorectal cancer progression. Carcinogenesis 2005;26:916–22.

49. Fukunari H, Iwama T, Sugihara K, Miyaki M. Intratumoral heterogeneity of genetic changes in primary colorectal carcinomas with metastasis. Surg Today 2003;33:408–13.

50. Loupakis F, Ruzzo A, Cremolini C, Vincenzi B, Salvatore L, Santini D, et al. KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. Br J Cancer 2009;101:715–21.

51. Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, et al. Colorectal cancer with mutation in BRAF, KRAS, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol 15;22:4584–94.

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