Clinical Relevance of KRAS-Mutated Subclones Detected with Picodroplet Digital PCR in Advanced Colorectal Cancer Treated with Anti-EGFR Therapy

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Abstract

Purpose: KRAS mutations are predictive of nonresponse to anti-EGFR therapies in metastatic colorectal cancer (mCRC). However, only 50% of nonmutated patients benefit from them. KRAS-mutated subclonal populations nondetectable by conventional methods have been suggested as the cause of early progression. Molecular analysis technology with high sensitivity and precision is required to test this hypothesis.

Experimental Design: From two cohorts of patients with mCRC, 136 KRAS, NRAS, and BRAF wild-type tumors with sufficient tumor material to perform highly sensitive picodroplet digital PCR (dPCR) and 41 KRAS-mutated tumors were selected. All these patients were treated by anti-EGFR therapy. dPCR was used for KRAS or BRAF mutation screening and compared with qPCR. Progression-free survival (PFS) and overall survival (OS) were analyzed according to the KRAS-mutated allele fraction.

Results: In addition to the confirmation of the 41 patients with KRAS-mutated tumors, dPCR also identified KRAS mutations in 22 samples considered as KRAS wild-type by qPCR. The fraction of KRAS-mutated allele quantified by dPCR was inversely correlated with anti-EGFR therapy response rate (P < 0.001). In a Cox model, the fraction of KRAS-mutated allele was associated with worse PFS and OS. Patients with less than 1% of mutant KRAS allele have similar PFS and OS than those with wild-type KRAS tumors.

Conclusions: This study suggests that patients with mCRC with KRAS-mutated subclones (at least those with a KRAS-mutated subclones fraction lower or equal to 1%) had a benefit from anti-EGFR therapies. Clin Cancer Res; 21(5); 1087–97. © 2014 AACR.

Introduction

Colorectal cancer is the third most common cancer worldwide with more than one million patients diagnosed each year, of which 50% will develop metastatic disease (1, 2). Recent efforts to improve the treatment of advanced metastatic colorectal cancer (mCRC) have led to the development of monoclonal antibodies, such as cetuximab or its fully humanized version, panitumumab, that inhibit the activation of the EGFR and its downstream pathways (namely RAS–RAF–MAPK and PI3K–PTEN–AKT) that promote cell growth, proliferation, and inhibition of apoptosis. Evidence shows, however, that patients with KRAS-mutant tumors receive little or no benefit from anti-EGFR therapies as single agents or combined with chemotherapy (3–6). These findings led the European Medicine Agency and the Food and Drug Administration to restrict the use of cetuximab and panitumumab to patients with wild-type KRAS tumors and, more recently, to NRAS wild-type tumors.

From studies including patients with apparently wild-type KRAS tumors, the response rates to cetuximab or panitumumab therapy ranged from only 40% to 60% (7), which results in a large fraction of patients without any known causes for treatment failure. The presence of alterations in other genes in the EGFR-dependent signaling pathways (8–10) is responsible for some of the nonresponding cases. However, these alterations do not account for all examples of primary or secondary resistance to anti-EGFR therapy. Recent works have demonstrated that secondary resistance to such therapies is associated with the emergence or selection of KRAS-mutated subclones in KRAS wild-type patients at the time of diagnosis (11). The presence of a low fraction of KRAS-mutated

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-14-0983
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cells within tumors, which is not detectable by commonly used procedures, could explain secondary resistances. Two hypotheses have been proposed. First, intratumor heterogeneity of KRAS mutations results in detection failures when analyzing a single biopsy (12). In addition, the limited sensitivity of conventional methods used for the detection of KRAS mutations leads to false-negative results (1, 5). Therefore, the performance of molecular analysis technologies to detect KRAS-mutant subclones at the time of diagnosis, and the consequence of subclone mutations for treatment of patients with mCRC are critically important. A highly sensitive and specific method of detection is necessary to probe the heterogeneity and the low prevalence of mutated DNA from the tumor. Furthermore, a quantitative detection technology allows analysis of response rate or progression-free survival (PFS) according to the absolute quantity of KRAS-mutant allele, which enables definition of a clinically relevant threshold.

Sanger sequencing offers a sensitivity of approximately 20% to detect KRAS-mutant alleles (13). This poor sensitivity has led to the use of alternative methods, such as pyrosequencing (14–16) or qPCR (3, 17), which have sensitivities ranging from 1% to 10% for the detection of mutant DNA in a background of wild-type DNA (18). Picodroplet digital PCR (dPCR) has recently emerged as a highly sensitive and quantitative approach for rare sequence detection (19). Droplet-based dPCR technology is based on parallel amplification of up to millions of individual DNA fragments within identical compartments (i.e., droplets), and sensitivity is limited only by the number of molecules that can be amplified and detected (i.e., the number of PCR-positive compartments) and the false-positive rate of the mutation detection assay. Previous reports demonstrate detection of one mutant KRAS gene in 200,000 wild-type KRAS genes in genomic DNA (20). A number of other examples of picodroplet dPCR for highly sensitive mutation detection have recently been published (21–23). In addition to high sensitivity, the ability to detect multiple mutations in a single experiment has also been demonstrated using picodroplet dPCR (20, 24, 25). The multiplex procedure has been adapted to the analysis of DNA integrity within formalin-fixed paraffin-embedded (FFPE) lung samples (25) and to the quantitative detection of the seven common mutations of KRAS (in codons 12 and 13) in plasma samples from patients with mCRC (26). In this report, we have extended this multiplex dPCR technology to investigate the incidence of low-frequency KRAS mutations in patients with mCRC and the consequence of these mutations on the response to anti-EGFR therapy.

Patients and Methods

Patients

Two cohorts of patients were pooled: one from an already published retrospective series (8) and one from a prospective series, “CETRAS study,” approved by the Ile-de-France ethical committee number 2 (ID-RCB 2007-AO124-49; AFSSAPS A70310-31). We selected all patients with KRAS, NRAS, and BRAF wild-type tumors (n = 136) for whom we have collected enough tissues to perform highly sensitive picodroplet dPCR and targeted next-generation sequencing. We added to this selection of triple-negative patients, 41 patients with KRAS-mutated tumors selected from the retrospective series for whom we had sufficient tumor material. Table 1 summarizes the clinical and pathologic characteristics of the patients.

All patients were refractory to FOLFOX and/or FOLFIRI regimen and were treated with anti-EGFR antibodies with or without chemotherapy. The different regimens are described in Table 1. The mean age was 62 years ± 11, and the gender ratio (M/F) was 1.33. The outcome measures were based on RECIST, PFS, and overall survival (OS).

Tumors were collected at time of surgery. For the 177 patients, 206 tissues samples were available either as frozen samples (121) or FFPE tissues (85). Tumor cell content was assessed by hematoxylin–eosin–safran (HES) staining. For paraffin-embedded tissues, manual selection of tumor-rich area was performed on HES-stained slides, followed by manual dissection of serial unstained slides under ×10 magnification. For frozen tissue, an HES staining procedure was also performed on different fragments and that containing the highest fraction of tumor cells was retained for DNA extraction. Among the tumor samples, 87% had a tumor cell content of 50% or more, and the rest of the tumor samples contained between 20% and 40% of tumor cells. KRAS and BRAF mutation statuses were assessed by allelic discrimination using Taqman qPCR probes as previously described (3, 8), and NRAS status was assessed by Sanger sequencing. All patients included were BRAF and NRAS wild-type.

Patients' characteristics (n = 177) Cases (%) Percentages

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Statistical analysis

A binomial negative law was used to estimate the number of KRAS wild-type patients that need to be included in the study to be able to detect, with 95% of confidence, 30 patients with a KRAS-mutated subclone. The expected occurrence frequency of tumors containing KRAS-mutated subclones used for the calculation was 30%; we were conservative from previous studies, which estimate this percentage between 38% and 60% from a small series (11, 27). Consequently, with this hypothesis, following a binomial negative law, it is required to test at least 130 tumors previously determined as KRAS wild-type by qPCR. We tested 136 tumors to take into account some potential technical issues.

The Kruskal–Wallis test was used to calculate the P value for association between the fraction of tumors containing KRAS-mutated allele and cetuximab response. PFS was calculated as the period from the first day of cetuximab treatment to the date of the tumor progression, to the date of death from any cause, or to the date of the last follow-up, at which point data were censored. OS was calculated as the period from the first day of cetuximab treatment until death from any cause or until the date for the last follow-up, at which point data were censored. A Cox model was used to estimate the effect of the fraction of KRAS-mutated DNA on survival. Both PFS and OS were estimated using the Kaplan–Meier method and compared using the log-rank test. A Cox model was used to estimate the effect of the fraction of KRAS-mutated allele(s) on survival. A receiver operating characteristics (ROC) analysis was performed to estimate a potential clinically relevant threshold for KRAS-mutated allele fraction. Analysis was carried out using STATA (STATA Corp.). The level of significance was set up at P = 0.05.

Probes and primers design

The seven common KRAS mutations on codons 12 and 13 were assessed as previously described (3, 25, 26). BRAF primers and probes were described elsewhere (22). The BRAF c.1797T>A (p. V600E) mutation detection was assessed by allelic discrimination using Taqman probes following the same protocol as for the KRAS mutations. Primers and probes were procured through ABI. Taqman probes targeting wild-type sequence are conjugated to VIC (from ABI, λem 528 nm)/λex 494 nm, and probes targeting mutant sequences are labeled with 6-carboxyfluorescein (6-FAM, λem 494 nm/λex 522 nm).

DNA controls

Genomic DNA was extracted from commercial cell lines purchased from the American Type Culture Collection (ATCC CCL-231, ATCC CRL-5807, ATCC CCL-255, ATCC CCL-227, ATCC CRL-5877, ATCC HTB-53, and ATCC CCL-229). The heterozygous cell line bearing the c.34G>C (p.G12R) KRAS mutation was described previously (25). Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen) and fragmented to an average size of 3,000 bp using the S2 Focused Ultrasonicator (Covaris). DNA concentration was determined using a Nanodrop ND-1000 spectrophotometer.

Positive controls were prepared by mixing 175 ng of sheared wild-type genomic DNA (gDNA; typically from Promega) with 1.75 to 3.5 ng (1%–2%) of sheared gDNA isolated from each cell line. Positive controls for the 4-plex KRAS assay included DNA from three cell lines, and the 5-plex assay included DNA from the other four cell lines. Positive control samples for the BRAF assay were prepared by mixing wild-type DNA with plasmid DNA containing the BRAF c.1799T>A (p.V600E) mutation. The appropriate concentration of plasmid DNA was determined empirically to yield a mixture in which the number of copies of mutant DNA was approximately 1% to 2% of the number of wild-type BRAF fragments.

DNA preparation

When using genomic DNA extracted from tumor cell lines, fragmentation of template DNA is necessary to decrease viscosity of the solution and allow rapid and stable droplet formation. This step was not required for DNA extracted from FFPE samples. However, for DNA extracted from frozen samples, it was necessary to use a heat fragmentation step to ensure proper shearing of the genomic DNA. Briefly, frozen DNA was submitted to a 10-minute 95°C denaturation step followed by a 15-minute incubation at room temperature before analysis.

KRAS and BRAF mutation detection

The duplex assay is based on the parallel amplification of wild-type and specific mutant sequences. In a pre-PCR environment, 12.5 µL Taqman Universal Master Mix (Life Technologies) was mixed by vortexing with the assay solution containing 0.75 µL of 40 mmol/L DNTIP Mix (New England BioLabs), 0.5 µL of 25 mmol/L MgCl2, 2.5 µL of Droplet Stabilizer (RainDance Technologies), 2.5 µL of 10× Taqman Assay Mix containing 8 µmol/L of forward and reverse primers, 2 µmol/L of 6-FAM, and 2 µmol/L of VIC. Taqman labeled target DNA template to a final reaction volume of 25 µL.

Assays for the detection of the seven mutations of KRAS located within codons 12 and 13 were assembled into two multiplex panels (a 4- and 5-plex dPCR assay) by mixing mutation-specific VIC and/or 6-FAM Taqman probes with a single wild-type (VIC) probe and a single pair of PCR primers in each panel. Complete description of the droplet-based procedures, including assay linearity and dynamic range, used in this study is described elsewhere (26).

All samples were assessed for the presence of the most frequent mutations of KRAS using the multiplex assay (26). The BRAF c.1797T>A (p.V600E) mutation was screened using a duplex assay (26).

Data analysis

Sensitivity of droplet-based PCR procedures is limited only by the number of molecules that can be amplified. As described earlier (26), the limit of detection of our assay is based on the limit of blank (LOB), and LOB is defined by the frequency of positive droplets measured in negative controls or controls with no DNA present. On the basis of these controls, the number of false-positive droplet events (i.e., LOB) for each of the seven KRAS assays is: 2 for c.34G>C (P.G12R); 2 for c.35G>C (P.G12D); 1 for c.34G>T (P.G12C); 8 for c.35G>C (P.G12A); 2 for c.34G>A (P.G12S); 11 for c.35G>T (P.G12V); 7 for c.38G>A (P.G13D) and 2 for the c.1797T>A (p.V600E) BRAF assay. The sample analysis was performed following the procedure described earlier for the same assays (26). The samples presenting low fraction of mutated allele(s) or ambiguous results (i.e., presenting droplet cluster with very low amounts of droplets close to LOB or non clustered population of droplets) were submitted to the appropriate duplex assay(s) to confirm the presence of the mutation(s). Only samples appearing positive in both experiments were classified as positives. The others were classified as negatives.
Tumor DNA samples generally present a relatively small fraction of amifiable DNA in particular in FFPE tumor samples (25), and therefore the measurement of amifiable DNA molecules is an important metric. Among the frozen samples, the amifiable DNA in the KRAS dPCR experiments ranged from 2,588 to 139,215 haploid genomes (mean = 37,329; median = 34,889). Among the FFPE samples, the amifiable DNA ranged from 342 to 49,096 (mean = 8,076; median = 3,059).

Sequence enrichment and sequencing
DNA samples extracted from frozen tissues with less than 16 μL total volume were normalized to a volume of 16 μL with the addition of 10 mmol/L Tris pH 8.0 (Sigma). Each 16 μL fresh-frozen DNA sample was fragmented by heating to 95°C for 10 minutes in an Mastercycler pro S (Eppendorf). DNA extracted from paraffin-embedded tissues was not submitted to such treatment because they are already fragmented by the formalin fixation.

Primary PCR reactions. All fresh-frozen samples had sufficient material to input 100 ng of DNA per reaction (measurement of DNA based on the number of amifiable genomes of each sample as previously determined by dPCR). When possible, 100 ng of FFPE was included in each PCR reaction. Each assay mix contained 6.5 μL of 10× High Fidelity PCR Buffer (Life Technologies), 1.75 μL of 50 mmol/L MgSO4, 2.25 μL of 10 mmol/L dNTPs, 5 μL of 4 mol/L Betaine, 5 μL of Droplet Stabilizer 10×, 2.5 μL of DMSO, 5 μL of 5 μmol/L BRAFT primers (5 μmol/L/primer). 5 μL of 5 μmol/L KRAS primers (5 μmol/L/primer), 1 μL (5 units/μL) of Platinum High Fidelity Taq polymerase in a total volume of 50 μL.

The PCR primers for KRAS and BRAFT amplicons were designed with the UCSC Browser. The sequences were as follows:

KRAS forward: CGCTCCTCCGATCCTCTGCTGTCCGTGACCAGTTAA TATGC, KRAS reverse: TGGCTCTTCGCCAGTACCCTGCTGACTAATGACT GAAT, BRAFT forward: CGCTCCTCCGATCCTGTTGCAATGTCCGTAATGCTGCT GCATT.

Each 50-μL reaction was dropletized on the RainDrop Digital PCR Source using standard procedures. The amplification was carried out in a Mastercycler pro S (Eppendorf) as follows: 2 minutes at 94°C, 55 cycles of (30 seconds at 94°C, 30 seconds at 54°C, and 60 seconds at 68°C), and finally 10 minutes at 68°C. After thermal cycling, 70 μL of droplet destabilizer (Raindance Technologies) was added to each sample, briefly vortexed and spun in a microcentrifuge. After addition of 2 μL of 3 mol/L NaOAc pH 5.2, the aqueous portion of each sample was purified using the Qiacube Automated Sample Prep System in conjunction with the standard MinElute PCR Purification Kit/Protocol and an elution volume of 15 μL.

Secondary PCR and cleanup. When possible, 10 ng of primary PCR product was included in each secondary PCR reaction; however, for samples where there was little or no amplification in the primary PCR reaction as detected on the Agilent Bioanalyzer, a total of 13 μL of the primary PCR reaction was added. The secondary PCR reaction mix (25 μL) was prepared as follows: 10× High Fidelity PCR Buffer (Life Technologies) 3.25 μL; MgSO4 (50 mmol/L) 0.875 μL; dNTPs (10 mmol/L) 1.125 μL; Betaine (4 mol/L) 2.5 μL; DMSO 1.25 μL; Secondary PCR primers (5 μmol/L/primer) 2.5 μL; primary template (10 ng); Platinum High Fidelity Taq polymerase 0.5 μL (5 units/μL); H2O to a total volume of 25 μL.

The sequences of the PCR primers used in the secondary PCR reaction were as follows:

### Secondary PCR reverse primer index sequences

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The amplification was carried out in a Mastercycler pro S (Eppendorf) as follows: 2 minutes at 94°C, 10 cycles of (30 seconds at 94°C, 30 seconds at 56°C, and 60 seconds at 68°C), and finally 10 minutes at 68°C. After addition of 2 μL of 3 mol/L NaOAc pH 5.2, each sample was then purified using the Qiacube Automated Sample Prep System in conjunction with the standard MinElute PCR Purification Kit/Protocol and an elution volume of 15 μL.

Sequencing. Using 10 mmol/L Tris-HCl pH 8.0, 1 μL of each sample was diluted to a concentration of 2 nmol/L. In contrast to quantifying the primary PCR products for secondary PCR, all material produced by the secondary PCR amplification—not just the approximately 300-bp target peak—was considered when diluting the sample for sequencing. An equal portion of each diluted sample was combined to produce a 2 nmol/L pool containing 48 total samples each with a different six-base index.

Sequencing was performed on an Illumina MiSeq instrument with the 500 cycle kit (part #: MS-102-2003) using the standard protocol for 250 base-paired end reads with the exception that custom sequencing primers, as described below, were substituted for the standard Illumina sequencing primers.

The sequences of the custom sequencing primers were as follows:

Read 1 custom sequencing primer (5′−3′) 5′-GATAGG.

BRAFT forward: CGCTCCTCCGATCCTCTGCTGTCCGTGACCAGTTAA TATGC, BRAFT reverse: TGGCTCTTCGCCAGTACCCTGCTGACTAATGACT GAAT.

The sequences were as follows:

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Clinical Relevance of KRAS-Mutated Subclones

Figure 1. Responses to anti-EGFR-based therapies and KRAS mutational status as determined by conventional procedures (qPCR) or droplet-based dPCR.

Read 2 sequencing primer (5'–3') loaded in MiSeq Cartridge Well #20: GTGACTGAGTGCTACGGTGCTTCCGATCTGAC.

The custom sequencing primers are identical to the standard Illumina sequencing primers except for the addition of 3 bases on the 3' end (shown in red) which dictate the end of the amplicon to which the secondary PCR primers will hybridize and extend.

Results

Of the 177 patients included in this study, 61 were responders; in one case, the response was not evaluable, and the others were not responders (Fig. 1). Tumor DNA extracted from this series was analyzed with picodroplet dPCR. In addition, part of KRAS exon 2 and BRAF exon 15 from 167 of these patients were analyzed with deep-next-generation sequencing (Table 2 and Supplementary Table S1).

All tumors were analyzed by multiplex dPCR targeting the seven mutations of the KRAS oncogene (see Supplementary Materials). This multiplex assay has been described elsewhere including assay linearity and dynamic range (26). First (Fig. 2, left), all the tumors previously highlighted as positive for KRAS mutations by qPCR (n = 41) were detected as positive with dPCR, and the percentage of KRAS-mutated allele ranges from 6.8% to 64.2% (median, 35.7%). Second, of the 136 tumors in which no mutations were detected with qPCR, 22 were found to be positive for a KRAS mutation by analysis with the multiplex dPCR assay. The percentage of KRAS-mutated allele(s) in this set of 22 samples ranges from 0.01% to 12.4% (median, 0.04%). Moreover, among all 177 samples, five tumor samples presented one or more additional low-frequency alleles that were only detected by dPCR (Table 2 and Fig. 2, right).

Tumor DNA from 163 patients, of the 177 of the series, was also analyzed by duplex dPCR to detect the c.1799T>A (p. V600E) mutation of the BRAF oncogene. Two tumors presented the BRAF c.1799T>A (p. V600E) mutation with fractions of mutated DNA of 6.9% and 0.07% (Table 2). The sample from patient 20, which contains 6.9% of mutated BRAF allele, also contains KRAS-mutated DNA, which was only detected by picodroplet dPCR. This sample presented a low fraction of three different KRAS mutations [i.e., less than 0.1% of c.38G>A (p.G13D), c.35G>A (p.G12D), and c.34G>A (p.G12S) DNA, which was verified with duplex dPCR analyses].

For 19 patients, at least one additional tumor sample was available (Supplementary Table S2). For all patients except for 53 and 493, samples from the same patients presented comparable results even for the presence of low-frequency alleles. For patient 53, the FFPE sample presented a low-frequency c.35G>A (p.G12D)–mutated clone (0.26%), which was not detected in the frozen sample from the same patient. Similarly, for patient 493, three samples were available, and one sample appears nonmutated, but two other samples presented a low-frequency c.35G>A (p.G12D) subclone (0.42% and 0.73% of c.35G>A (p.G12D)–mutated allele). The difference in the fraction of mutated alleles among samples can be explained by genetic heterogeneity within the whole tumor.

One hundred sixty-seven samples were enriched for KRAS and BRAF by single-molecule picodroplet dPCR, and then sequenced with next-generation sequencing. KRAS and BRAF mutation status of the samples (positivity threshold was set up at 1% for next-generation sequencing [NGS] results) was in agreement with dPCR results for 97% of the samples (162/167, see Supplementary Table S1). We observed a correlation with the observed fraction of mutant alleles with the two procedures (r² = 0.8 for all detected alleles).

We observed an inverse correlation between the proportion of mutated DNA and the frequency of anti-EGFR response (Fig. 3, P < 0.001). The mean percentage of mutated DNA was 0.45% and 12.5% for responders and nonresponders, respectively. Only 2 patients were classified as responders with a KRAS-mutant allele fraction greater than 10%. In a first analysis, we considered the percentage of mutant allele as a continuous variable. PFS and OS in a Cox model showed that the percentage of KRAS-mutant allele is significantly associated with worse PFS and OS. Considering an incremental of 1% mutant allele, the HRs were 1.03 [95% confidence interval (CI), 1.02–1.04; P < 0.001] and 1.02 (95% CI, 1.01–1.03; P < 0.001) for PFS and OS, respectively, with adjustment for age, gender, and number of chemotherapy lines received before anti-EGFR therapy.
<table>
<thead>
<tr>
<th>Sample names</th>
<th>Tumor mutation status (qPCR analysis)</th>
<th>Tumor mutation status (qPCR analysis)—main allele</th>
<th>Fraction of mutated allele—main clone (%)</th>
<th>Fraction of other mutated alleles (%)</th>
</tr>
</thead>
<tbody>
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<td>c.38G&gt;A (p.G12D) (0.01); c.35G&gt;A (p.G12S) (0.02)</td>
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<td>c.38G&gt;A (p.G13D)</td>
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<td>Not detected</td>
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<td>6.9</td>
<td>c.38G&gt;A (p.G13D) (0.04); c.35G&gt;A (p.G12D) (0.02); c.34G&gt;A (p.G12S) (0.02)</td>
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<tr>
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<td>c.38G&gt;A (p.G13D)</td>
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</tr>
<tr>
<td>303</td>
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<td>c.35G&gt;A (p.G12D)</td>
<td>13.6</td>
<td>Not detected</td>
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<td>Not detected</td>
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<tr>
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</tr>
<tr>
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<td>c.38G&gt;A (p.G13D)</td>
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<tr>
<td>383</td>
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<td>c.1799T&gt;A (p.V600E)</td>
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<td>Not detected</td>
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<tr>
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<td>c.35G&gt;A (p.G12D)</td>
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<tr>
<td>470</td>
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<td>c.34G&gt;T (p.G12V)</td>
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<td>c.34G&gt;C (p.G12R)</td>
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<tr>
<td>473</td>
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<td>c.35G&gt;T (p.G12V)</td>
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<tr>
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<td>NM</td>
<td>c.35G&gt;A (p.G12D)</td>
<td>0.01</td>
<td>Not detected</td>
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</table>
In a second analysis, we categorized patients into quartiles of KRAS-mutant allele fraction (cutoff of 0.57%, 21.3%, and 40.3%). We observed an inverse association between the quartile and the response rate. Among the 63 patients with at least one positive test (i.e., qPCR or dPCR), the response rate was evaluable for 62. Among them, the 12 patients that were responders belong to the first two quartiles, whereas 31 of the 50 nonresponders belong to the third and fourth quartiles. This difference is statistically significant ($P < 0.001$). Furthermore, a Kaplan–Meier survival curve showed an inverse correlation between PFS or OS and the categorization of samples into the different quartiles ($P < 0.001$). Furthermore, patients in the lowest quartile have comparable PFS and OS to those patients without detectable mutations (Fig. 4A and B).

We tried to determine a potential clinically relevant threshold for the ratio of mutant allele. We estimated the diagnostic value (response rate) of the fraction of KRAS-mutated alleles by performing an ROC curve analysis. The area under the curve was 0.89. The optimal value of KRAS-mutated alleles was 1.5% with 87% of correctly classified patients for response status (i.e., responders vs. nonresponders). We therefore choose the value of 1% to dichotomize the patients with no mutations detected by qPCR ($n = 136$). There is evidence that patients with a tumor mutated with more than 1% of a mutant allele have a significantly worse prognosis than those negative by both qPCR and dPCR or those with less than 1% mutant allele fraction. When comparing patients with nonmutated tumors or with tumors presenting 1% or less of a mutated allele with patients with tumor with more than 1% of mutated allele, the HRs were 3.2 (95% CI, 1.3–7.9; $P = 0.014$) and 4.7 (95% CI, 1.6–14.3; $P = 0.006$) for PFS respectively and 4.1 (95% CI, 1.6–10.4; $P = 0.003$) and 5.6 (95% CI, 1.8–16.8; $P = 0.002$) for OS (Fig. 4C and D). Furthermore, considering that 5 patients had a fraction of KRAS-mutated allele between 1% to 10%, the relevant clinical cutoff value cannot be precisely defined and is reasonably between 1% and 10%; therefore, we can estimate that usual standard procedures of KRAS-mutant detection missed, at the maximum, 3.6% of clinically relevant KRAS mutation (95% CI, 1.2–8.3), and therefore this percentage accounts for unexplained resistance in "KRAS wild-type patients."

### Discussion

In this work, the high sensitivity and precision of picodroplet dPCR enabled detection and quantification of rare KRAS and BRAF-mutated alleles in tumor samples with the aim of understanding the importance of subclones for patient treatment management. Indeed even if KRAS mutations have been described as the major predictive factor for resistance to anti-EGFR responses, a large proportion of patients (40%–60%) with a tumor classified as nonmutated do not respond to such therapies (7).

Many research studies have been conducted to try to understand and overcome these treatment failures, including strategies aimed at finding mutations in other genes of the EGFR-dependent signaling pathways (8–10). However, except for extended RAS mutations (10), none of the candidate genes get through clinical validation. Another approach has been to focus on developing and applying new, highly sensitive procedures to detect low-frequency KRAS alleles and make correlations to clinical data to understand the importance of the rare mutations. Various studies have highlighted that sensitive molecular analysis methods would allow clinicians to identify all patients that will not benefit from Cetuximab (1, 28–30). For example, in the largest series published (1, 29), the authors used sensitive methods of detection (pyrosequencing or MALDI-TOF MS, mutant-enriched PCR, and engineered mutant-enriched PCR) to demonstrate that the patients with mutations identified by methods more sensitive than direct sequencing are more likely to have a lower response rate or worse PFS.

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**Table 2. Analysis of the patient mutation status (Cont'd)**

<table>
<thead>
<tr>
<th>Sample names</th>
<th>Tumor mutation status (qPCR analysis)</th>
<th>Tumor mutation status (dPCR analysis)—main clone</th>
<th>Fraction of mutated allele—main clone (%)</th>
<th>Fraction of other mutated alleles (%)</th>
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<td>X to Y</td>
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<td>NM</td>
<td>Not detected</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

NOTE: Tumors have been classified based on the measured fraction of the primary detected mutated allele. Supplementary Tables S1 and S2 summarize the data obtained for each patient.
These series compared different methods of KRAS detection with Sanger sequencing, which is known to present low sensitivity for the detection of specific mutation. Furthermore, the question raised by these studies is the lower limit of detection, which has a clinical relevance to give some clinical guidance, underlining the need of truly quantitative methods leading to robust results and to alleviate possible experimental bias.

Droplet-based dPCR, which employs thousands to millions of aqueous droplets to compartmentalize a DNA sample, has emerged as a promising strategy for the analysis of clinical samples (26, 31–33). dPCR is both precise and sensitive due to discrete counting of the mutant and wild-type alleles present in a sample (34, 35). The sensitivity is only limited by the number of molecules that can be analyzed and the false-positive rate of the mutation detection assay. Another advantage of microfluidic droplet-based procedures is the possibility to quantify the amount of amplifiable DNA in tested samples. This quantification corresponds to an internal positive control of the assay and allows definition of the limit of detection attainable for this particular sample (25).

In this study, we used picodroplet digital procedures (see ref. 19 for a review) to highlight low-frequency KRAS or BRAF-mutated clones as previously described (20, 22, 26). In addition to 41 KRAS-mutated tumors that were included as positive controls, 136 tumor samples that were characterized by qPCR as negative for KRAS, BRAF, and NRAS mutations were analyzed by picodroplet dPCR. As expected, the procedure enabled detection of all tumors with a KRAS mutation identified by conventional procedures (n = 41). Among the 136 remaining tumors, 22 were positive for a KRAS mutation (16%) and two were positive for the BRAF c.1799T>A (p.V600E) mutation based on dPCR analysis. The fraction of subclones observed in KRAS "wild-type" patients generally agrees with the fraction of patients with mCRC exhibiting emergence of KRAS-mutated clones in their plasma during anti-EGFR treatments. Diaz and colleagues (11) highlighted that 38% (9/24; 95% CI, 19–59) of patients with a tumor initially classified as wild-type developed detectable mutations in KRAS in serum analyzed during or after treatment. Moreover, this study revealed 3 patients that presented multiple KRAS mutations in their blood. The occurrence of multiple subclones after treatment by anti-EGFR therapy is also consistent with the number observed in our study (6 patients with multiple subclones in their tumors). The slightly lower percentage of mutated patients observed in our study is expected because circulating tumor blood DNA is a better sampling of the tumor heterogeneity than that from a small part of the primary tumor (36).

As described in previous studies, nearly all patients with KRAS-mutated tumor samples that were identified by bulk procedures were nonresponders (40/41, 97.5%). However, among the newly identified KRAS-mutated tumors, half of them (11/22) belong to responder patients. Furthermore, we observed an inverse correlation between the proportion of mutated DNA and the frequency of anti-EGFR response (P < 0.001). If we consider all patients positive for a mutation, 75% of the patients in the lowest quartile were responders, 25% of the patients in second quartile were responders, and none of the patients in the upper two quartiles were responders. Using an ROC analysis, the cut-point allowing the highest percentage of correctly classified patients is 1.5%. If we...
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Figure 4.
Correlation between the fraction of mutated KRAS alleles in the tumor and patient survival. A, PFS and (B) OS of the 177 patients of the study divided in four quartiles based on the detected fraction of KRAS alleles. C, PFS and (D) OS of the 136 patients detected as wild-type by conventional qPCR methods as a function of the fraction of KRAS alleles. NM, nonmutated.

consider a cutoff value of 1%, only 2 patients (out of 46) with more than 1% of KRAS-mutant allele were responders as compared with 62.5% of the patients with 1% or less of KRAS-mutant allele. This association with response was translated in terms of PFS and OS. The quantitative association between survival and mutant KRAS allele concentration was assessed by the Cox model. The results reveal a direct link between these 2 variables treated as continuous variables, which suggests a direct mechanism between treatment and time to emergence of resistant subclones driven by the selection of pre-existing mutated subclones (11). However, the low number of patients with a fraction of KRAS-mutated allele between 1% and 10% (5) does not allow to determine a clinically relevant cutoff with a strong confidence between these values. Large prospective studies, ideally from clinical trials, are strongly needed to translate these findings in clinical setting.

Furthermore, with the development of new highly sensitive technologies, it is now necessary to ensure clinical pertinence of mutant allele detection. By using quantitative method, we are able to suggest clinically relevant threshold helping to decide or not to treat patients with mCRC with anti-EGFR therapies. The remaining variability is likely due to the variation of quantification of tumors cells in the studied samples, which have been enlightened in some studies (37). A bias induced by a high level of the tumor fragment contamination by normal cells will increase the number of nonresponders in the group of patients with low fraction of KRAS-mutated allele.

The ability to distinguish, quantify, and then monitor individual subclones is one of the appealing benefits of droplet-based dPCR procedures. Tumors are composed of various cell populations with distinct mutations, and treatment will act as a selection pressure on these multiple clones by promoting the selection of a particular cell population (38). After an initial response, resistance invariably occurred, limiting the clinical benefit of the treatment (27). A highly sensitive procedure, such as dPCR, facilitates tracking of individual clones, which would enable truly personalized medicine by customizing therapy regimens to delay disease recurrence.

Disclosure of Potential Conflicts of Interest

P. Laurent-Puig is a consultant/advisory board member for Merck Serono, Amgen, and RainDance Technologies. D.R. Link has ownership interest (including patents) in RainDance Technologies. O. Bouché has received bureau honoraria from Amgen and is a consultant/advisory board member for Merck Serono. V. Boige has provided expert testimony for Merck Serono. V. Taly is a consultant/advisory board member for RainDance Technologies. No potential conflicts of interest were disclosed by the other authors.

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Clin Cancer Res; 21(5) March 1, 2015 1095

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Grant Support

D. Pekin was supported by a fellowship from the "Region Alsace." This work was supported by the Ministère de l’Enseignement Supérieur et de la Recherche, the Université Paris-Descartes, the Université de Strasbourg, the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSEM), the Institut National du Cancer (INCA; no. 2009-1-RT-03-US-1 and 2009-RT-03-IIPS-1), the Association pour la recherche contre le cancer (ARC; no. SL2010061375), the Agence Nationale de la Recherche (ANR Nanobiotechnologies; no. ANR-10-NANO-0002-09), and the SIRIC CARPÉM and canceropole funding (no. 2011-1-LABEL-IUPS-2).

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Received April 21, 2014; revised July 15, 2014; accepted July 19, 2014; published OnlineFirst September 23, 2014.

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www.aacrjournals.org Clin Cancer Res; 21(5) March 1, 2015 1097

Published OnlineFirst September 23, 2014; DOI: 10.1158/1078-0432.CCR-14-0983
Clinical Relevance of *KRAS*-Mutated Subclones Detected with Picodroplet Digital PCR in Advanced Colorectal Cancer Treated with Anti-EGFR Therapy

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