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Low Percentage of *KRAS* Mutations Revealed by Locked Nucleic Acid Polymerase Chain Reaction: Implications for Treatment of Metastatic Colorectal Cancer

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Metastatic colorectal cancer (mCRC) is frequently characterized by the presence of mutations of the *KRAS* oncogene, which are generally associated with a poor response to treatment with anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies. With the methods currently used, a case is classified as *KRAS*-mutated when approximately 20% of the cells bear an activating *KRAS* mutation. These considerations raise the question of whether cells with a mutated *KRAS* can be found in mCRC cases classified as *KRAS* wild-type when more sensitive methods are used. In addition, the issue arises of whether these mCRC cases with low proportion of *KRAS*-mutated cells could account at least in part for the therapeutic failure of anti-EGFR therapies that occur in 40–60% of cases classified as *KRAS* wild type. In this study, we compared the classical assays with a very sensitive test, a locked nucleic acid (LNA) polymerase chain reaction (PCR), capable of detecting *KRAS*-mutated alleles at extremely low frequency (detection sensitivity limit 0.25% mutated DNA/wild-type DNA). By analyzing a cohort of 213 mCRC patients for *KRAS* mutations, we found a 20.6% discordance between the sequencing/TheraScreen methods and the LNA-PCR. Indeed, 44 mCRC patients initially considered *KRAS* wild type were reclassified as *KRAS* mutated by using the LNA-PCR test. These patients were more numerous among individuals displaying a clinical failure to anti-EGFR therapies. Failure to respond to these biological treatments occurred even in the absence of mutations in other EGFR pathway components such as *BRAF*.

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INTRODUCTION

In recent years, improvements in the treatment of metastatic colorectal cancer (mCRC) have prompted a significant increase in patient overall survival. Among new therapies, monoclonal antibodies (mAbs) targeting epidermal growth factor receptor (EGFR), which is expressed at the surface of neoplastic cells, have represented an important step forward in the management of mCRC (1–3). These antibodies block antiapoptotic/proliferative signals delivered by the EGFR to malignant cells. Such a mechanism is supposed to operate only in the cells that have functional EGFR-dependent signal transduction pathways, since mutations in genes encoding proteins located downstream of EGFR may cause constitutive activation of this pathway (4). Indeed, it has been demonstrated that in patients with mutated *KRAS* or, to a lesser extent, *BRAF* genes, the response to the two available antibodies to EGFR (panitumumab or cetuximab) is low or absent (5–9). On the basis of these observations, and with the aim of increasing the efficacy of treatment while avoiding unnecessary toxicity, the European Medical Agency and the U.S. Food and Drug Administration defined a *KRAS* mutation test that is mandatory before deciding for or against therapy with mAbs (10,11).

Technical guidelines for the *KRAS* assay were issued by the European Society of Pathology and the College of American Pathologists (12,13). Sanger direct sequencing, real-time polymerase chain reaction (PCR) and pyrosequencing are all considered suitable, although none are recommended specifically. A 3–5% detection limit of mutant alleles

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can be achieved by the integrated use of these methods (14,15).

The sensitivity issue is relevant, particularly in the case of samples containing relatively small fractions of malignant cells, where the mutated allele is possibly confounded within the "background" noise range of the normal cells unless macrodissection or microdissection laser capture is used (16). Moreover, not all of the malignant cells may bear the *KRAS* mutation, and this phenomenon may be attributed to the intratumor genetic heterogeneity that is frequently observed in solid tumors (17,18), including mCRC (19,20). Consequently, the presence of low levels of *KRAS*-mutated alleles in tumors classified as *KRAS* wild type by routine methods may influence the response to anti-EGFR therapy and would provide an explanation for the short-response duration of certain mCRC patients to cetuximab/panitumumab treatment. Indeed, such targeted treatment may favor *KRAS*-mutated cell growth (21).

On such premises, the present study was undertaken with the aim of searching for correlations between the presence of *KRAS*-mutated subclones in *KRAS* wild-type tumors and the clinical response of patients to cetuximab treatment.

MATERIALS AND METHODS

Patient Population

A total of 213 patients with a histologically confirmed diagnosis of metastatic colorectal cancer were recruited from a cohort of 450 mCRC patients referred to our laboratory for the diagnostic determination of *KRAS* mutational status between January 2009 and June 2011.

The following criteria had to be fulfilled for patient selection: (a) availability of suitable formalin-fixed paraffin embedded (FFPE) tissues, (b) higher than 75% cancer cellularity in the samples to be investigated and (c) high-quality and sufficient quantity of DNA for further analyses (see below). The main clinical characteristics of patients are summarized in Table 1.

Clinical records were available for 95 patients among the 129-patient *KRAS* wild-type population. All patients were treated with cetuximab in combination with chemotherapy-based regimens (FOLFIRI regimen in 27 patients, FOLFOX regimen in 17 patients and irinotecan in 51 patients). Cetuximab was administered at a loading dose of 400 mg/m^2 (milligrams/square meters) over 2 h, followed weekly by 250 mg/m^2 over 1 h. A total of 51 patients received one prior line of chemotherapy treatment, and 44 received more than two treatment lines. Treatment was continued until disease progression or intolerable toxicity. Clinical response was assessed every 6–8 wks by radiological examination (lung and abdomen CT scan). The Response Evaluation Criteria in Solid Tumors, version 1.0 (RECIST v1.0) (22), was adopted for clinical evaluation, and objective response was classified as complete response, partial response, stable disease (SD) or progressive disease (PD). Patients were classified as responders if they achieved complete response or partial response and nonresponders if they demonstrated stable SD or PD.

The study was carried out according to the principles of the Declaration of Helsinki and was approved by our institutional ethical committee. Written informed consent for molecular analyses was obtained from all patients.

Table 1. Characteristics of mCRC patients.

	KRAS wild-type	*KRAS* mutated
n	129	84
Age, median (range)	62 (39–86)	77 (37–88)
Sex		
M	74	46
F	55	38
Primary tumor site		
Colon	107	73
Rectum	22	11
Tissue for *KRAS* analysis		
Primary	105	69
Metastases	24	15

Samples and DNA Extraction

FFPE tumor samples from mCRC patients were selected from individuals undergoing routine *KRAS* genotyping. A pathologist performed tumor macrodissection on tissue blocks to enrich for neoplastic tissue. In addition, hematoxylin-eosin–stained sections of those selected tissues were examined by the same pathologist to determine whether tumor cellularity was above the prespecified threshold of 75%. Only samples fulfilling these criteria were used for subsequent studies.

Genomic DNA was extracted from five FFPE 10-μm-thick sections and from the colon cancer cell lines HCT 116 and Colo 320 by using the QIAamp DNA mini-kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. In each sample, quality and purity of isolated DNA were evaluated by 0.8% agarose gel electrophoresis and by the calculation of the 260/280 spectrophotometric ratio by using the Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA).

Cell Lines and Sensitivity Tests

The HCT 116 cell line harboring a heterozygous *KRAS* codon 13 mutation (GGC>GAC) and the Colo 320 cell line, which is *KRAS* wild type, were obtained from the American Type Culture Collection (ATCC) (distributed by LGC Standards, Sesto San Giovanni, Milan, Italy). Dilutions for sensitivity studies were performed by mixing the DNA extracted from HCT 116 cells with that extracted from Colo 320 cells. The amount of DNA extracted from both the cell lines was first measured by determining the *GAPDH* housekeeping gene concentrations by real-time PCR and a standard curve of placental DNA (Sigma-Aldrich, Milano, Italy). Subsequently, mutated DNA was added to wild-type DNA in percentages ranging from 50% to 0.125% (mutated DNA/wild-type DNA).

KRAS Mutational Analysis

All patient samples were screened for *KRAS* codons 12 and 13 mutations for diagnostic purpose by standardized and validated operating procedures consisting of an integrated PCR/Sanger sequencing and real-time PCR approach, as indicated

by current international recommendations (23,24). Briefly, 100–200 ng genomic DNA was amplified by PCR by using 2.5 U Taq Gold DNA polymerase (Life Technologies, Carlsbad, CA, USA), 1× buffer, 2 mmol/L $MgCl_2$, 200 nmol/L dNTP, and 30 pmol forward and reverse primers in a final volume of 50 μL. Two different sets of primers (*KRAS* external [ext] forward: 5′ GTG TGA CAT GTT CTA ATA TAG TCA and *KRAS* ext reverse: 5′ GAA TGG TCC TGC ACC AGT AA; *KRAS* 172 forward: 5′ GGC CTG CTG AAA ATG ACT GAA T and *KRAS* 172 reverse: 5′ TCA AAG AAT GGT CCT GCA CC) were used to amplify the region spanning the codon 12 and 13 of *KRAS* exon 2 and produce amplicons of two different sizes (212 and 172 bp, respectively). The amplified PCR products were then treated with ExoSap (GE Healthcare, Waukesha, WI, USA) as recommended and both strands were sequenced by dye terminator cycle sequencing (BigDye Terminator v3.1; Life Technologies). The nucleotide sequence detection was performed on an ABI Prism 3130 Genetic Analyzer (Life Technologies) according to standard protocols. The sequence data were analyzed by using MacVector software, version 11 (MacVector, Cary, NC, USA), to identify mutations and to assign genotypes to individual DNA samples. A *KRAS* mutation was assigned only when at least three-fourths of the sequences from two independent PCR amplifications gave the same result.

The second method used for the detection of *KRAS* mutations was the commercially available TheraScreen®: K-RAS Mutation Kit (Qiagen, Hilden, Germany). This kit allows the detection of the six codon 12 mutations and one codon 13 mutation in the *KRAS* gene. Samples were amplified according to the manufacturer's protocol and analyzed by using the OneStep Plus PCR System (Life Technologies).

Mutational Analysis of *KRAS* by High-Sensitivity PCR Locked Nucleic Acid–Clamped Probe Real-Time Assay

Mutations at codons 12 and 13 in the *KRAS* gene were detected by the real-time PCR locked nucleic acid (LNA)-clamped probe (LNA-PCR) (LightMix *KRAS* kit; TIB Molbiol, Berlin, Germany). This is a real-time PCR assay used for the search of *KRAS* mutations in codons 12 and 13 by amplification of the *KRAS* gene and a subsequent melting curves analysis with hybridization FRET probes (a sensor probe, 12-Cys specific, and an anchor probe). Amplification is realized in the presence and/or absence of a competitor, an LNA oligomer spanning the codon 12 and 13 wild-type region that achieves the inhibition of the wild-type *KRAS* allele, allowing preferential amplification of mutated ones. Amplification of 100 ng genomic DNA was run on a LightCycler PCR machine, and fluorescence data were analyzed by using the LightCycler software (Roche Diagnostics, Monza, Italy). After PCR run, melting curves (and thus, the melting temperature [Tm]) were generated for detection and identification of the sample *KRAS* mutation. Mutational analysis for each tumor sample was performed at least twice. LNA-PCR products that gave a melting curve corresponding to a *KRAS* mutation were directly sequenced to confirm and to define the type of *KRAS* mutation.

Molecular Cloning

Molecular cloning assay was performed on three different mCRC cases. *KRAS* PCR was performed as described above. After PCR, the amplicons were cloned into the TOPO TA vector according to the manufacturer's instructions (Life Technologies). A range of 110–130 individual colonies were picked up and plasmid DNA isolated with the NucleoSpin plasmid (Machery-Nagel, Duren, Germany) were sequenced by BigDye Terminator 3.1 (Life Technologies) by using standard sequencing primers M13.

Mutational Analysis of BRAF by Direct Sequencing

BRAF exon 15 was amplified and sequenced by the Sanger method as above to detect V600 mutation as previously described (25).

Statistical Analysis

Fisher exact test and χ^2 tests were used as appropriate to compare proportions of responders and nonresponders according to their *KRAS* mutational status. Statistical significance was set up to a p value of <0.05.

All supplementary materials are available online at www.molmed.org.

RESULTS

Determination of the Analytical Sensitivity of Three Different *KRAS* Mutation Detection Methods

We first compared the detection limit of the Sanger direct sequencing method and the TheraScreen *KRAS* mutation kit (which are both used in routine clinical practice) with that of the LNA-PCR method, so far used only for research purposes. DNA extracted from the cells of the HCT 116 cell line harboring the GGC>GAC *KRAS* mutation was added to wild-type DNA from the cells of the Colo 320 line in different proportions ranging from 50% to 0.125% (see Materials and Methods for details). With the Sanger direct sequencing method, the GGC>GAC *KRAS* mutation was readily detectable when the mutated DNA was 12.5%, although a lower-intensity peak corresponding to the *KRAS* mutation was still discernible at the 6.25% dilution (Figure 1A). On the basis of these observations, it was concluded that about 10% of *KRAS* mutant alleles are readily detected by the sequencing method in DNA containing predominantly wild-type *KRAS*. With the TheraScreen kit, used within the same range of dilutions, the sensitivity was about 4% of the mutant allele (Figure 1B). In conclusion, an integrated procedure on the basis of the Sanger sequencing method and TheraScreen: K-RAS Mutation Kit had an overall true detection limit of approximately 4% mutated DNA/wild-type DNA.

In contrast with the LNA-PCR assay, a melting curve and the Tm of 57.6°C (corresponding to the presence of GGC>GAC *KRAS* mutation) were still visualized

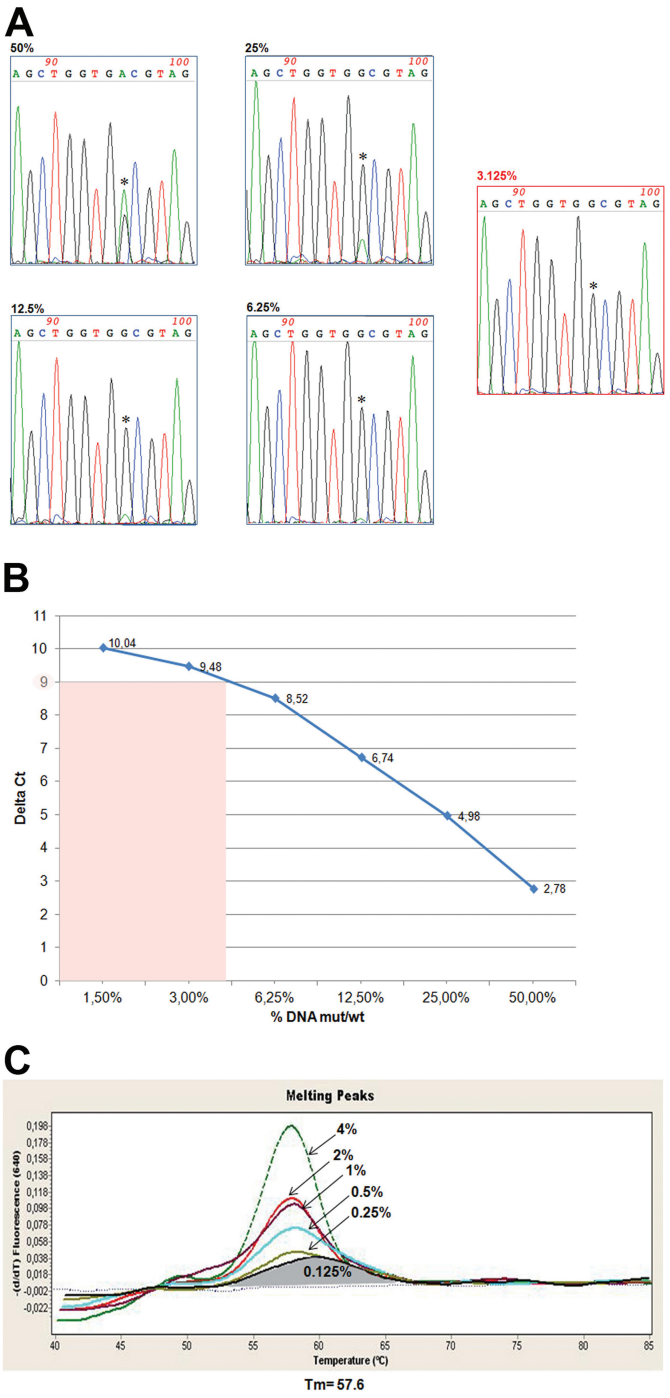

Figure 1. Sensitivity threshold of PCR/sequencing, TheraScreen and LNA-PCR assays. DNA from HCT 116 cells carrying the gac codon 13 *KRAS* mutation (G13D) was diluted with the *KRAS* wild-type DNA from Colo 320 cells in proportions ranging from 50% to 0.125% mutant DNA. (A) Sequencing electropherograms of a 212-bp amplicon are reported for the different DNA mixtures indicated. (B) Correlation between ΔCt values and dilutions of mutant DNA in a TheraScreen kit, ΔCt: difference between Ct of mutation assay and Ct of control assay. The blue diamond indicates the 1% cutoff ΔCt value for the G13D probe (corresponding to the 9 value). (C) The melting profiles corresponding to the gac codon 13 *KRAS* change (Tm = 57.6°C) are generated with the mutated DNA/wt DNA dilutions (range from 4% to 0.125%) by the LightCycler software.

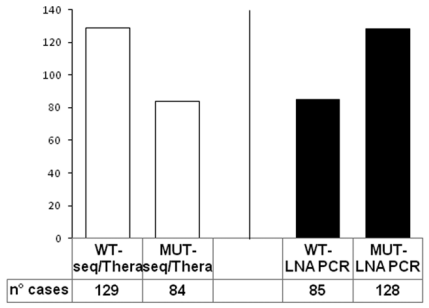

Figure 2. Comparison of mutation frequencies detected by sequencing/TheraScreen and the LNA-PCR methods. Distribution of *KRAS*-mutated and *KRAS* wild-type mCRC cases with sequencing/TheraScreen (left) and the LNA-PCR assays (right).

when mutated *KRAS* represented the 0.25% of total DNA (Figure 1C). Notably, to demonstrate the presence of the gac *KRAS* mutation at the highest mutant dilutions used in the LNA-PCR assay, and to gather a further specificity proof, a nucleotide sequence was generated directly from the amplified products of the LNA-PCR–clamped probe real-time PCR assay. The analysis of the sequences of the amplification products confirmed the presence of gac mutation in each of the dilutions tested from 4% to 0.25% (data not shown).

KRAS Mutation Analysis in mCRC Cases by Direct Sequencing/TheraScreen Methods

KRAS genotyping was performed on tissues from primary tumor (174 of 213) or metastatic sites (39 of 213). Mutated *KRAS* was detected in 84 of 213 (39.4%) cases by direct sequencing and TheraScreen kit, whereas wild-type *KRAS* was observed in 129 of 213 (59.6%) cases (Figure 2). The *KRAS* mutations observed were those reported previously, with substitutions at codon 12 more frequent than those at codon 13 (79.8% versus 20.2%). Gly12Asp and Gly12Val mutations were the most frequent at codon 12, accounting for the 47.8% and 23.9%, respectively, followed by the Gly12Ala (14.9%) and Gly12Ser (9%) changes of the total. The remaining three mutations (4.4%) involving codon 12 sub-

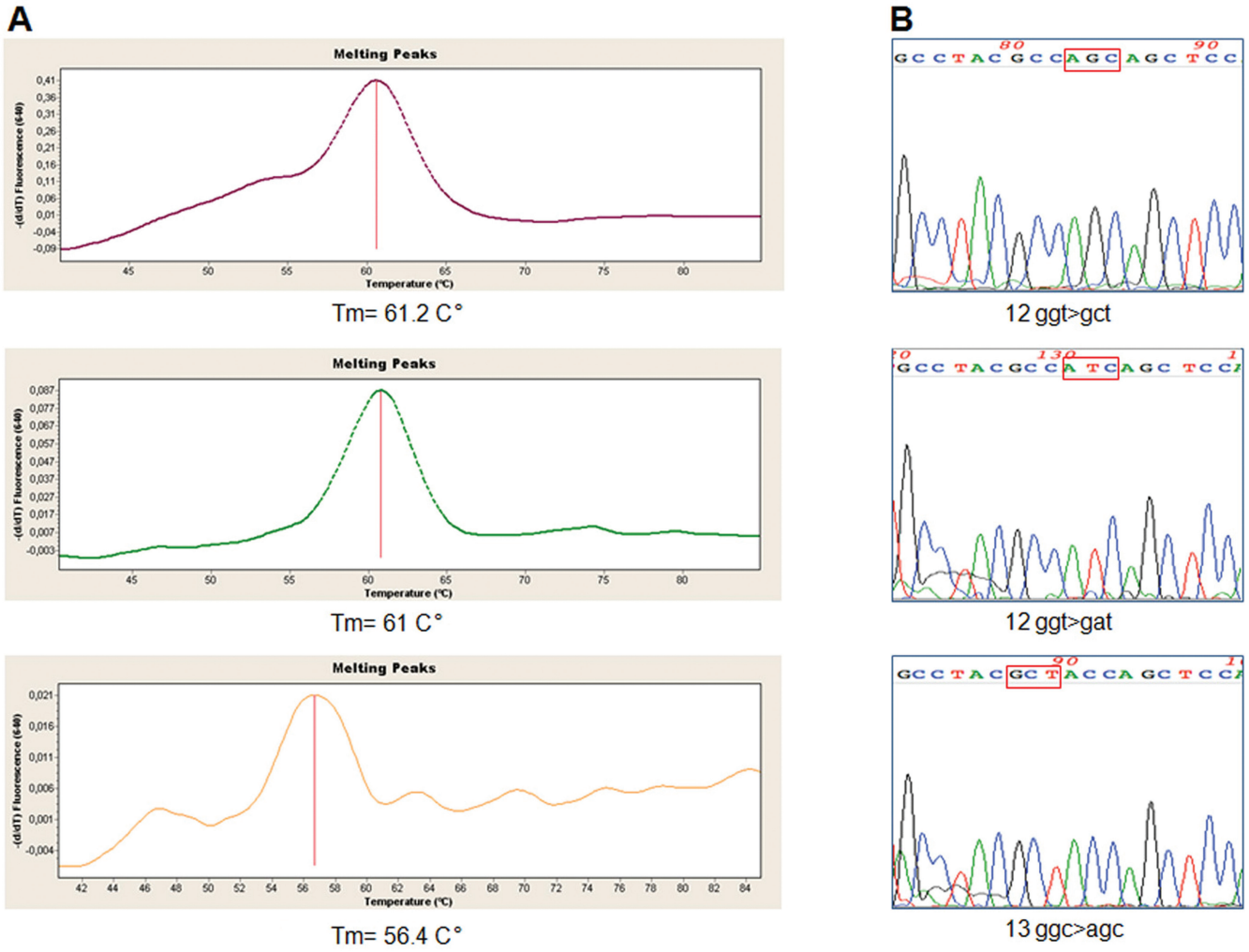

Figure 3. Representative cases with mutations in *KRAS* gene detected by using the LNA-PCR method. Melting curves (A) and corresponding sequence electropherograms (B) obtained from LNA-PCR–amplified products of three different mCRC cases are shown.

stitutions were represented by Gly12Arg (one mutation) and Gly12Cys (two mutations) change. Within the codon 13 mutations, the majority of them (15 cases) exhibited the classical Gly-to-Asp change (13D), and only 2 of 17 displayed the less frequent Gly-to-Cys amino acid change (13C). Only 1 of 213 cases exhibited a two-nucleotide variation at the same codon 12. Notably, the percentage of cases discordant with the sequencing or the TheraScreen kit was 2.35%. In three of five cases, the DXS kit helped the identification of *KRAS* mutation, which resulted as an unreadable mutated peak with direct sequencing; whereas in the two others, the cells had the Gly13Cys mutation that was recognized only by sequencing (Supplementary Table S1).

Identification of Low-Frequency *KRAS* Mutated Alleles in mCRC Cases by LNA-PCR

All 213 mCRC cases were investigated by using the LNA-PCR. By using this approach, 44 cases had discordant results compared with the sequencing/TheraScreen methods. Consequently, the number of patients resulting in *KRAS* wild type and *KRAS* mutated were re-quoted in 85 of 213 (39.9%) and 128 of 213 (60.1%), respectively (Figure 2, right columns). A total of 44 cases initially classified as *KRAS* wild type by the sequencing/TheraScreen methods could be reclassified as mutated by the LNA-PCR method. Instead, none of the cases classified as mutated by sequencing/TheraScreen (84 cases) turned out to be wild-type by the LNA-PCR, indicating an equal reproducibility but a greater sensitivity for the LNA-PCR assay.

In the 44 cases who proved positive for the *KRAS* mutation by LNA-PCR (and wild-type for sequencing/TheraScreen), the amplification products were directly sequenced. This approach confirmed the presence of mutations in all of the cases reclassified as mutated. The relevant chromatograms of three representative cases are reported in Figure 3. Notably,

Table 2. Summary of clinical response in relation to *KRAS* mutational status.

	RP/RC	SD	PD	Total
All	29 (30.5%)	37 (38.9%)	29 (30.5%)	95
WT/WT	23 (36.5%)	22 (34.9%)	18 (28.6%)	63
WT/mutated	6 (18.75%)	15 (46.85%)	11 (34.4%)	32

the distribution of *KRAS* mutations in this subgroup of patients was similar to that of mutations detected by less sensitive methods (26) (that is, mutations were mainly in the codon 12 [36 of 44, 81.8%], whereas eight cases presented substitutions in codon 13 [18.2%]) (not shown). Among the eight mutations at codon 13, three resulted in G13D, three in G13S and two in G13C changes, respectively (Supplementary Table S1). Among the G12D-expressing cases (in total 19 of 44), two have an additional mutation in codon 13 (G13D and G13S, respectively).

To confirm the presence of rare *KRAS*-mutated alleles (detected only by LNA-PCR methodology), a 212-bp *KRAS* gene region, which encompasses the 12 and 13 codons, from three patient specimens was amplified and cloned. One, two and four *KRAS* mutant clones were detected in 125, 110 and 130 molecular clones, from the three different samples, respectively, which were classified *KRAS* wild type by conventional methods (not shown). These experiments also confirm the low percentage of mutations in these patients.

On the basis of the above results, analyzed cases were grouped as follows: patients with *KRAS* wild type by both sequencing/TheraScreen and LNA-PCR (called WT/WT) and patients with *KRAS* wild type by sequencing/TheraScreen but *KRAS*-mutated according to the LNA-PCR method (called WT/MUT).

Clinical Response to Anti-EGFR mAb Therapies of the Two WT/WT and WT/MUT Subgroups of mCRC Patients

Subsequently, we analyzed whether the presence of low proportions of cells with a *KRAS*-mutated allele within the tumor mass might have some clinical impact in terms of response to treatment with anti-EGFR mAbs. The clinical records were available for 95 patients, 63 of whom were WT/WT cases and 32 of whom were WT/MUT cases. Among the WT/WT patients, a response to therapy was observed in 36.5% (23 of 63) of cases, whereas this response occurred in 18.75% (6 of 32) of the WT/MUT cases. Collectively, nonresponders (SD + PD) patients were 63.5% and 81.25% in the WT/WT and WT/MUT subgroups, respectively (Table 2). In the present setting, a difference between these two groups was only marginally significant (Fisher and χ^2 tests, $p = 0.059$ and $\chi^2 = 3.16$, respectively).

Recently, it was reported that the G13D *KRAS* mutation defines a subgroup of patients with different tumor biology and clinical outcomes. The *KRAS* G13D–bearing patients seem to respond to anti-EGFR treatment therapy in a similar manner as patients with *KRAS* wild type (27,28). In our study, among the WT/MUT subgroup, two cases who responded to biological treatments showed the classical G13D mutation, whereas two additional cases expressed the rarest G13S and G13C, respectively. All of these G13X-expressing cases seemed to benefit from anti-EGFR therapy. Interestingly, when the G13D-mutated cases were excluded from the statistical computation, a significant difference was observed in terms of response to biological therapy ($p = 0.017$ and $\chi^2 = 5.3$) between WT/WT and WT/MUT subgroups as defined above.

Analysis of the BRAF V600 Mutation

Because of the potential *BRAF* involvement in resistance to anti-EGFR therapy, *BRAF* mutational status was assessed in all the 213 mCRC cases studied. Fourteen patients of 213 mCRC cases (6.6%) presented the V600 *BRAF* mutation. All the *BRAF* V600–positive cases were within the WT/WT subgroup, and none were present in the WT/MUT cases (Supplementary Table S1). This observation demonstrates that even when very sensitive techniques capable of detecting mutations of *KRAS* in small neoplastic subclones are used, *KRAS* and *BRAF* mutations are mutually exclusive, in agreement with systems biology modeling of cancer network modules (29). Among the eight *BRAF*-mutated patients, three had a partial response to anti-EGFR therapies, whereas the remaining five cases were nonresponders.

DISCUSSION

In this study, we first explored the issue of whether subclones exhibiting mutated *KRAS* alleles could be detected among the mCRC cases initially classified as *KRAS* wild type.

To this end, two routine methods, direct sequencing and TheraScreen system, were compared with an LNA-PCR technique. Whereas the detection limit of both sequencing and TheraScreen were closed to those reported for the two tests in the literature, with some interlaboratory variation (26), LNA-PCR reached a much higher sensitivity threshold (about 0.25% mutated DNA/wild-type DNA), making this test suitable and reliable for identifying low frequency of *KRAS*-mutated alleles. In our cohort of 213 patients, both sequencing and TheraScreen methods were highly concordant, displaying only a 2.4% discordance. In contrast, the LNA-PCR methodology was capable of detecting 44 *KRAS* additional mutations, raising the proportion of *KRAS* mutated cases in the cohort from 40% to 60%. Considering the results of all the specificity tests performed, including those of molecular cloning of *KRAS* in three different individuals, our findings lead to the conclusion that a small fraction of tumor cells expressing mutated *KRAS* alleles can be present in an apparently wild-type *KRAS* tumor. Whether this low amount of mutant *KRAS* alleles reflects the presence of different *KRAS*-expressing subclones remains a speculation, primarily because

other biases, such as stromal tissue contamination or inflammatory cells, may in part contribute to this event. Nevertheless, to limit the effects of these biases, FFPE tissues were reviewed by a pathologist, and areas containing at least 75% neoplastic cells were selected. In connection with this, it is of note that Arcila *et al.* (30), by using an LNA-based PCR technique, could also detect 18% additional *KRAS* mutants compared with direct sequencing in 168 metastatic tissues from mCRC patients. This percentage is similar to ours, which was obtained by analyzing mainly the cells from primary tumors, since 7 of 44 WT/MUT cases were only classified as such because of data on metastatic tissue in our cohort. Furthermore, recently, the appearance of *KRAS* mutant molecules was demonstrated in the plasma of mCRC patients after anti-EGFR treatment and interpreted as related to the presence of *KRAS*-mutated clones originating early in oncogenesis (31).

Two patients from the WT/MUT subgroup who responded to anti-EGFR therapy displayed the *KRAS* G13D mutation. A statistically different response between WT/MUT and WT/WT subgroups to anti-EGFR treatment was observed by excluding these cases from computations. Therefore, our data seem to support the emerging body of evidence that not all the *KRAS* mutations have the same weight in tumor biology and patient clinical outcome and that patients with *KRAS* G13D mutant tumors may behave as *KRAS* WT patients (27,28).

BRAF V600E alterations were not found among the WT/MUT subgroup (but were found solely in the WT/WT cases). Interestingly, a fraction of patients with *BRAF* mutation still responded to anti-EGFR therapy, although the number of observations is too low to provide any conclusive indication.

Recently, Santini *et al.* (32) reported that the presence of a very low frequency of *KRAS*-mutated subclones does not affect the response to anti-EGFR mAbs on the basis of the retrospective analysis of a relatively small cohort of patients. The authors conclude that a highly sensitive test could exclude potentially responsive patients from anti-EGFR therapy. This conclusion is in contrast to our observations and to the similar conclusions reached by Molinari *et al.* (33) in a recent report published while our study was in preparation. The combined findings of our studies and those by Molinari's group suggest that a higher-sensitivity *KRAS* genotyping method may enhance the predictive value of this biomarker, with important consequences on the cost-to-benefit ratio of anti-EGFR treatments in the mCRC setting. These conclusions justify the efforts to improve the sensitivity of the methods to detect *KRAS* mutations.

A final topic to be discussed relates to the time of appearance of *KRAS* mutations. Recently, Diaz *et al.* (31) suggested that preexisting *KRAS*-mutated subclones may be the mediator of acquired resistance to EGFR blockade in mCRC by applying a mathematical modeling. This conclusion is in line with the results of the present study, although an alternative hypothesis should be considered (that is, that in the WT/MUT cases, the *KRAS* mutations may be a *de novo* late acquisition that occurs only in a minority of subclones). If this is the case, these cells may have some growth advantage, but it is uncertain whether they would eventually outgrow the remaining wild-type subclones.

CONCLUSION

We have shown that rare subclones with *KRAS* mutations within a wild-type *KRAS* tumor are detectable with high-sensitivity methods such as LNA-PCR methods and may in part account for the inaccuracy of prediction of anti-EGFR therapy efficacy. These low-prevalence *KRAS* mutations may indeed identify tumors with poorer response to EGFR-targeting mAbs.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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