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
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/m² (milligrams/square meters) over 2 h, followed weekly by 250 mg/m² 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.

**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

| 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              |
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 three times. DNA extracted from the cells of the HCT 116 cell line harboring the GGC>GAC mutation kit had an overall true detection rate of approximately 4% mutated alleles. With the Sanger direct sequencing method, the GGC>GAC mutation was readily detectable when the mutated DNA was 12.5%, although a lower-intensity peak corresponding to the presence of GGC>GAC mutation was still discernible at the 6.25% dilution (Figure 1A).

RESULTS

Statistical Analysis

Fisher exact test and χ² 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.
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-
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 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 shown in Figure 3. Notably,
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 $\chi^2$ 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

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

<table>
<thead>
<tr>
<th>Status</th>
<th>Total</th>
<th>RP/RC</th>
<th>SD</th>
<th>PD</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>1524</td>
<td>29 (30.5%)</td>
<td>37 (38.9%)</td>
<td>29 (30.5%)</td>
<td>95</td>
</tr>
<tr>
<td>WT/WT</td>
<td>44</td>
<td>23 (36.5%)</td>
<td>22 (43.2%)</td>
<td>18 (28.6%)</td>
<td>63</td>
</tr>
<tr>
<td>WT/Mutated</td>
<td>32</td>
<td>6 (18.75%)</td>
<td>15 (46.85%)</td>
<td>11 (34.4%)</td>
<td>32</td>
</tr>
</tbody>
</table>
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.

REFERENCES

11. Cetuximab (Erbitux) and panitumumab (Vectibix); KRAS mutations [Internet]. Silver Spring (MD): U.S. Food and Drug Administration; [updated 2010 Jan 11; cited 2013 Jan 22]. Available from: http://www.fda.gov/AboutFDA/Centers.


