The Oncologist®

Gastrointestinal Cancer

High Concordance of *KRAS* Status Between Primary Colorectal Tumors and Related Metastatic Sites: Implications for Clinical Practice

DANIELE SANTINI,[a] FOTIOS LOUPAKIS,[b,c] BRUNO VINCENZI,[a] IRENE FLORIANI,[d] IRENE STASI,[b] EMANUELE CANESTRARI,[e] ELIANA RULLI,[d] PAOLO ENRICO MALTESE,[b] FRANCESCA ANDREONI,[e] GIANLUCA MASI,[b] FRANCESCO GRAZIANO,[f] GIACOMO GIULIO BALDI,[b] LISA SALVATORE,[b] ANTONIO RUSSO,[g] GIUSEPPE PERRONE,[h] MARIA ROSA TOMMASINO,[i] MAURO MAGNANI,[e] ALFREDO FALCONE,[b,c] GIUSEPPE TONINI,[a] ANNAMARIA RUZZO[e]

[a]Department of Oncology and [h]Surgical Pathology, University Campus Bio-Medico, Rome, Italy; [b]Department of Oncology, Azienda USL6 Livorno, Istituto Toscano Tumori, Italy; [c]Department of Oncology, Transplants and New Technologies in Medicine, University of Pisa, Pisa, Italy; [d]Oncology Department, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy; [e]Dipartimento di Scienze Biomolecolari, University of Urbino, Urbino, Italy; [f]Division of Medical Oncology, Azienda Ospedaliera San Salvatore, Pesaro, Italy; [g]Sezione di Oncologia Medica, Dipartimento di Discipline Chirurgiche ed Oncologiche, and [i]Dipartimento di Patologia Umana, Università di Palermo, Palermo, Italy

Key Words. Colorectal cancer • *KRAS* mutations • Concordance • Metastatic sites • Primary tumors

LEARNING OBJECTIVES

After completing this course, the reader should be able to:

1. Describe the importance of *KRAS* mutations in CRC patients.
2. Explain the relevance to cancer treatment of concordance of *KRAS* status between primary tumors and metastases in CRC patients.
3. Discuss the impact of *KRAS* mutations as a predictive/prognostic factor in CRC patients.

CME This article is available for continuing medical education credit at CME.TheOncologist.com.

ABSTRACT

Purpose. **Several studies have suggested that *KRAS* somatic mutations may predict resistance to cetuximab- and panitumumab-based treatments in metastatic colorectal cancer (CRC) patients. Nevertheless, most experiences were conducted on samples from primaries. The aim of this study was to evaluate the grade of concor-**

Correspondence: Daniele Santini, M.D., Ph.D., Medical Oncology, University Campus Bio-Medico, Via Alvaro del Portillo, 200, 00128 Rome, Italy. Telephone: 0039-06-225411206; Fax: 0039-06-225411934; e-mail: d.santini@unicampus.it Received August 13, 2008; accepted for publication October 28, 2008; first published online in ***THE ONCOLOGIST Express*** on December 4, 2008. 1083-7159/2008/$30.00/0 doi: 10.1634/theoncologist.2008-0181

dance in terms of *KRAS* status between primaries and related metastases.

Patients and Methods. **We analyzed *KRAS* codon 12 and 13 mutations from formalin-fixed sections of 107 CRC primaries and related metastases. Eight pairs were excluded from the analysis because of the low amount of tumor tissue in the available samples. The main characteristics were: 50 men, 49 women; median age at diagnosis, 71 years (range, 41–84). The metastatic sites analyzed were the liver in 80 patients (80.8%), lung in seven patients (7.1%), and other sites in 12 patients (12.1%).**

Results. **A *KRAS* mutation was found in 38 (38.4%) primary tumors and in 36 (36.4%) related metastases. The rate of concordance was 96.0% (95% confidence interval, 90.0%–98.9%). Discordance was observed in only four (4%) patients.**

Conclusions. **Our results indicate that the detection of *KRAS* mutations in either primary or metastatic tumors from patients with CRC is concordant and this assessment could be used to predict response to targeted therapies such as cetuximab and panitumumab.** ***The Oncologist*** 2008;13:1270–1275

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in western countries [1]. Recent therapeutic strategies are based on targeting the epidermal growth factor receptor (EGFR), which is overexpressed in CRC [2]. Cetuximab and panitumumab are monoclonal antibodies (mAbs) directed against EGFR. Blockade of EGFR disrupts downstream signaling pathways such as the phosphatidylinositol 3′ kinase/Akt, Ras/Raf/mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription pathways, all of which are crucial in the regulation of cell growth, proliferation, apoptosis, invasion, migration, and angiogenesis [1]. On the basis of the Bowel Oncology with Cetuximab Antibody (BOND) trial, cetuximab was approved [3] in European countries in combination with irinotecan for the treatment of patients with $EGFR^+$ metastatic CRC who progressed on a prior irinotecan-based chemotherapy regimen. In subsequent retrospective analyses, no correlation was found between EGFR expression assessed by immunohistochemistry (IHC) and clinical outcome [3, 4]. The need for alternative predictive factors has become imperative in order to avoid unnecessary toxicities and waste of resources.

KRAS mutations occur in about 40% of primary CRCs [5, 6], and such mutations have been demonstrated to be predictors of resistance to anti-EGFR mAbs. In the presence of specific mutations of the *KRAS* gene, the Ras protein, which has intrinsic GTPase activity, is constitutively activated, and subsequent signaling events are unregulated and independent from EGFR control [7]. Several studies [8–13] in patients with advanced or metastatic CRC have indicated that the presence of *KRAS* mutations (*KRAS* point mutations in codons 12 and 13) is associated with a lack of response to cetuximab.

More recently, in patients with *KRAS* mutations, panitumumab was demonstrated to be ineffective [14], and therefore this drug was granted approval for the treatment of patients with wild-type *KRAS* who have chemotherapy-refractory CRC. In all the cited studies [8–13], the mutational analysis was conducted almost exclusively on primary tumors.

It has been shown that primary colorectal tumors may differ from their corresponding metastases in terms of EGFR [15, 16], Akt, and MAPK protein expression assessed by IHC [17]. While it is well known that *KRAS* mutations occur in the first steps of colorectal carcinoma progression [18], additional data have demonstrated that the frequency of *KRAS* mutations in lymph node metastases is higher than in the related primary CRCs [19]. Because mutations in downstream targets of EGFR have been shown to correlate with a lesser response to the EGFR agent cetuximab, assessing this intracellular molecular target might help to predict response to such treatments. Therefore, it may be of primary importance to verify the correlation between primaries and related metastases with regard to *KRAS* status.

The primary endpoint of this study was to verify the concordance in terms of *KRAS* status between primary CRCs and paired metastatic sites.

Patients and Methods

Patient Selection

Patients were selected from a pathology database of CRC cases undergoing surgical resection of the primary tumor and surgical resection or diagnostic biopsy of the synchronous or metachronous corresponding metastatic site and collected in the pathology departments of the Azienda USL6 of Livorno, Università degli Studi di Pisa, Università Campus Bio-Medico, and Università degli Studi di Palermo between 1998 and 2007. All these patients had not been, at the time of specimen collection, treated with any targeted agents such as cetuximab and panitumumab.

KRAS Mutational Status

We searched for *KRAS* point mutations in codons 12 and 13, two hotspots that include >95% of mutations in this gene, as already reported [20].

Polymerase Chain Reaction Amplification

DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) CRC specimens using the Qiamp DNA FFPE tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In total, 25–50 ng of DNA was added to a volume of 25 μl with 150 μM deoxynucleotide triphosphates, 6.25 pmol of each primer, 1 U of Hot-Rescue DNA Polymerase (Diatheva, Fano, Italy), 5% dimethylsulfoxide, 2 mM $MgCl_2$, and 2.5 μl of 10× polymerase chain reaction (PCR) buffer. Primers spanned codon 12 and 13 of the *KRAS* gene. Primers for the 214-bp amplicon that spanned exon 2 were 5′-GTGTGACATGTTCTAATATAGTCA-3′ (forward) and 5′-GAATGGTCCTGCACCAGTAA-3′ (reverse). PCR cycling was run according to the following conditions: one cycle of 95°C for 10 minutes; 40 cycles of 95°C for 45 seconds, 56°C for 45 seconds, and 72°C for 45 seconds; and one cycle of 72°C for 10 minutes.

DNA Sequencing

PCR products were column purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions and eluted in a 15-μl volume. Concentrations were estimated with the ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The cleaned PCR product (10–15 ng) was then used as a template in cycle sequencing with the BigDye Terminator v.1.1 CycleSequencing Kit (Applied Biosystems, Foster City, CA). The reaction mix consisted of 1 μl Terminator ready reaction mix, 1× sequencing buffer, and 3.2 pmol sequencing primer in a 20-μl total volume.

Two sequencing reactions were performed for each template with the nested primer 5′-GCCTGCTGAAAATGACTGAA-3′ (forward) and 5′-TGAATTAGCTGTATCGTCAAGGCACT-3′ (reverse).

Reactions were run according to the following protocol: one cycle of 96°C for 2 minutes and 40 cycles of 96°C for 10 seconds, 55°C for 5 seconds, and 60°C for 2 minutes. Sequencing reactions were purified with the DyeEx 96 Kit (Qiagen) and run on an ABI PRISM 310 Genetic Analyser (Applied Biosystems). The sense and antisense strands were aligned and analyzed with Sequence Navigator Software (Applied Biosystems).

Statistical Analysis

Considering the primary endpoint, the level of concordance of *KRAS* status between primaries and related metastatic samples was stated at a minimum desirable of 90%, while it was not acceptable at a level <80%. Using the design proposed by A'Hern for the binomial distribution [21], setting the probability of erroneously concluding that the concordance is >80% at 5% (one-sided $\alpha = .05$) and the probability of correctly concluding that the concordance is at least 90% at 85% ($\beta = 0.15$), it was necessary to perform both evaluations in at least 94 patients. The minimum number of concordant samples was set at 82 of 94, because this result is associated with the lower limit of the 90% confidence interval (CI) of concordance of 80.1%.

Table 1. Patient characteristics

Characteristic	Value
Total *n* of patients	99
Age, median (range)	71 (41–84 yrs)
Gender, male/female	50/49
Primary tumor site	
Right colon	33
Left colon	46
Rectum	20
Histology	
Adenocarcinoma	89
Mucinous differentiation	10
Tumor grade	
1–2	63
3	36

Results

We retrospectively analyzed *KRAS* codon 12 and 13 mutations in primary tumors and related metastatic sites from 107 CRC patients. Eight pairs were excluded from the analysis because of the low amount of tumor tissue in the available samples. Ninety-nine patients were available for the final analysis (Table 1)—50 men (50.5%) and 49 women (49.5%); median age at diagnosis, 71 years (range, 41–84 years). Thirty-three were affected by right CRC, 46 by left CRC (including intraperitoneal rectal adenocarcinoma), and 20 by extraperitoneal rectal cancer. CRC adenocarcinoma was the unique histotype, and in 10 cases a mucinous differentiation was detected. Histologic grade 1–2 and grade 3 were described in 63 (63.6%) and 36 (36.4%) tumors, respectively.

In total, 99 pathologic samples from metastatic sites were analyzed. The most common site of origin of metastatic tissue was the liver, which was noted in 80 (80.8%) cases (synchronous in 75 cases and metachronous in the remaining 24 cases), and the lung, which was noted in seven cases (metachronous in five cases). The others organs of tumor origin in the remaining cases are shown in Table 2.

Table 2. Sites of metastasis

Site	*n*
Liver	80
Lung	7
Peritoneum	5
Nonregional lymph nodes	1
Bone	2
Local relapse	2
Ovary	2

Table 3. Codon distribution of specific *KRAS* mutations

	Primary tumor	Metastatic tissue
G12A	1	1
G12C	3	2
G12D	11	10
G12R	1	1
G12S	2	2
G12V	13	12
G13D	7	8

A *KRAS* mutation was found in 38 (38.4%) primary tumors and in 36 (36.4%) related metastatic sites. The grade of concordance between primaries and metastatic sites was 96.0% (95% CI, 90.0%–98.9%). Details regarding the frequencies of the different codon 12 and 13 *KRAS* mutations in this population are reported in Table 3. All patients, with the exception of one, with wild-type *KRAS* primaries also showed wild-type *KRAS* metastases. Discordance was observed in only four (4%) patients. In particular, in one patient *KRAS* was wild-type in the primary tumor and mutated in the metastatic site (peritoneum, metachronous); in three patients *KRAS* was mutated in the primary tumor and wild-type in the metastatic site (liver in all three cases, two synchronous and one metachronous).

Discussion

Metastatic CRC represents a major global health problem, but the introduction of a novel class of targeted antineoplastic agents, mAbs, directed against the EGFR has significantly changed the therapeutic options available for these patients. As EGFR targeting provides real advantages only in a small subgroup of patients, several attempts have been made to identify predictive factors of treatment benefit.

Positive IHC staining for EGFR has been used as a criterion for patient selection. All the reported studies, however, have failed to demonstrate any correlation between the efficacy of cetuximab and EGFR IHC staining [3, 4]. Nowadays, several retrospective studies have clearly demonstrated the high predictive value of *KRAS* mutations in metastatic CRC patients treated with anti-EGFR mAb-based therapy [8–13]. Because identification of the mutational status of *KRAS* could help to select patients who have a high probability of benefiting from anti-EGFR antibodies [14], it may be of primary importance to verify the degree of correlation between primaries and related metastases with regard to *KRAS* status.

In the present observational analysis, we clearly reported a very high concordance (96.0%; 95% CI, 90.0%–98.9%) between primaries and related metastases in terms of *KRAS* mutational status. A similar grade of concordance was reported by Molinari et al. [22], who analyzed *KRAS* mutational status in a small sample of 30 consecutive patients with CRC with synchronous or metachronous metastasis. The same mutational pattern between primaries and corresponding metastases was observed in 26 cases. In three cases the mutation was restricted to the primary, and in one case it was restricted to the metastatic lesion, with a total grade of concordance of 86.6%.

We believe that the data reported in this paper have to be considered to be of strong clinical impact. Notably, we included a very large sample of patients (99 patients) with the same clinical and pathological characteristics of those included in the clinical trials investigating cetuximab- and panitumumab-based therapies. Furthermore, the prevalence (36.4%) of *KRAS* mutations in our sample is similar to that reported in the literature (about 40%) [5, 6]. We excluded from the analysis patients with a low amount of available tumor tissue to avoid false-negative cases derived from the analysis of the mutational status of normal tissue. In addition, the mutational status of *KRAS* was analyzed in a double-blinded fashion for each primary and metastatic sample. In the few cases in which the mutational status was uncertain, this was interpreted by a third molecular biologist.

The correlation analysis between the primary cancer and corresponding metastatic site was discordant in only four cases. In particular, in one patient *KRAS* was wild-type in the primary tumor and mutated in the metastatic site; in three patients *KRAS* was mutated in the primary tumor and wild-type in metastatic site. This high grade of concordance confirms that *KRAS* mutation represents a very early mutational step in CRC pathogenesis and plays a central role in tumor progression. Indeed, *KRAS* mutations are commonly found in patients with CRC and in the near normal mucosa and, in general, this mutation

might represent an early, stable genetic event that precedes the appearance of histologically detectable aberrations in colonic epithelial cells [23].

Recent findings underline the importance of a true outcome predictor for patients with metastatic CRC who are treated with an anti-EGFR mAb. An improvement in terms of the response rate and progression-free survival time for the combination of cetuximab plus chemotherapy versus chemotherapy alone in the first-line treatment of metastatic CRC was recently demonstrated in a phase III randomized trial [24]. In this setting, the vascular endothelial growth factor (VEGF) inhibitor bevacizumab has already been approved [25] in combination with irinotecan, fluorouracil, and leucovorin for the treatment of metastatic CRC. In the near future, the selection of patients who really benefit from anti-EGFR mAbs will be even more imperative, especially for those with metastatic lesions, who are potential candidates for secondary resection in which tumor shrinkage is the major goal of treatment. For these reasons, future prospective studies may be aimed at evaluating the role of *KRAS* mutations in directing the choice of which biologic (anti-EGFR, anti-VEGF, or both) could be the best partner for upfront chemotherapy. At the same time, *KRAS* analysis may help to avoid unnecessary toxicities and to optimize the allocation of resources for treating patients in the second- or third-line settings. To our knowledge, this is the first report indicating that the analysis of any available neoplastic tissue (primary or metastatic) for *KRAS* status is to be considered adequate and reliable in the vast majority of patients and this will be of great importance in routine clinical practice.

Author Contributions

Conception/design: Daniele Santini, Fotios Loupakis, Francesco Graziano, Giacomo Giulio Baldi, Alfredo Falcone, Giuseppe Tonini
Financial support: Daniele Santini
Provision of study materials: Fotios Loupakis, Eliana Rulli, Paolo Enrico Maltese, Francesca Andreoni, Gianluca Masi, Lisa Salvatore, Antonio Russo, Giuseppe Perrone, Maria Rosa Tommasino, Mauro Magnani, Alfredo Falcone, Giuseppe Tonini, Annamaria Ruzzo
Collection/assembly of data: Bruno Vincenzi, Irene Stasi, Emanuele Canestrari, Eliana Rulli, Paolo Enrico Maltese, Francesca Andreoni, Gianluca Masi, Giacomo Giulio Baldi, Lisa Salvatore, Antonio Russo, Giuseppe Perrone, Maria Rosa Tommasino, Mauro Magnani, Alfredo Falcone, Giuseppe Tonini, Annamaria Ruzzo
Data analysis: Bruno Vincenzi, Irene Floriani, Francesco Graziano
Manuscript writing: Fotios Loupakis, Bruno Vincenzi, Irene Floriani, Francesco Graziano
Final approval of manuscript: Daniele Santini, Irene Stasi, Emanuele Canestrari, Gianluca Masi, Francesco Graziano, Antonio Russo, Maria Rosa Tommasino, Mauro Magnani, Alfredo Falcone, Giuseppe Tonini, Annamaria Ruzzo

References

1 Jemal A, Siegel R, Ward E et al. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.

2 Herbst RS, Shin DM. Monoclonal antibodies to target epidermal growth factor receptor-positive tumors: A new paradigm for cancer therapy. Cancer 2002;94:1593–1611.

3 Cunningham D, Humblet Y, Siena S et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 2004;351:337–345.

4 Chung KY, Shia J, Kemeny NE et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 2005;23:1803–1810.

5 Andreyev HJ, Norman AR, Cunningham D et al. Kirsten *ras* mutations in patients with colorectal cancer: The multicenter "RASCAL" study. J Natl Cancer Inst 1998;90:675–684.

6 Andreyev HJ, Norman AR, Cunningham D et al. Kirsten *ras* mutations in patients with colorectal cancer: The 'RASCAL II' study. Br J Cancer 2001;85:692–696.

7 Bos JL. *ras* oncogenes in human cancer: A review. Cancer Res 1989;49:4682–4689.

8 Lièvre A, Bachet JB, Le Corre D et al. *KRAS* mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 2006;66:3992–3995.

9 Lièvre A, Bachet JB, Boige V et al. *KRAS* mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. J Clin Oncol 2008;26:374–379.

10 Di Fiore F, Blanchard F, Charbonnier F et al. Clinical relevance of *KRAS* mutation detection in metastatic colorectal cancer treated by cetuximab plus chemotherapy. Br J Cancer 2007;96:1166–1169.

11 Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F et al. Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res 2007;67:2643–2648.

12 De Roock W, Piessevaux H, De Schutter J et al. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. Ann Oncol 2008;19:508–515.

13 Khambata-Ford S, Garrett CR, Meropol NJ et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 2007;25:3230–3237.

14 Amado RG, Wolf M, Peeters M et al. Wild-type *KRAS* is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008;26:1626–1634.

15 Scartozzi M, Bearzi I, Berardi R et al. Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: Implications for treatment with EGFR-targeted monoclonal antibodies. J Clin Oncol 2004;22:4772–4778.

16 Cappuzzo F, Finocchiaro G, Rossi E et al. EGFR FISH assay predicts for response to cetuximab in chemotherapy refractory colorectal cancer patients. Ann Oncol 2008;19:717–723.

17 Scartozzi M, Bearzi I, Berardi R et al. Epidermal growth factor receptor (EGFR) downstream signalling pathway in primary colorectal tumours and related metastatic sites: Optimising EGFR-targeted treatment options. Br J Cancer 2007;97:92–97.

18 Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–532.

19 Oliveira C, Velho S, Moutinho C et al. *KRAS* and *BRAF* oncogenic mutations in MSS colorectal carcinoma progression. Oncogene 2007;26:158–163.

20 Frattini M, Balestra D, Suardi S et al. Different genetic features associated with colon and rectal carcinogenesis. Clin Cancer Res 2004;10:4015–4021.

21 A'Hern RP. Sample size tables for exact single-stage phase II designs. Stat Med 2001;20:859–866.

22 Molinari F, Martin V, Bordoni A et al. Analysis of epidermal growth factor receptor (EGFR) gene status and protein expression, and K-*ras* gene mutations in metastatic colorectal cancer patients: Comparison between primary tumor and related metastatic sites. Ann Oncol 2008;19(suppl 1):i10–i25.

23 Zhu D, Keohavong P, Finkelstein SD et al. K-*ras* gene mutations in normal colorectal tissues from K-*ras* mutation-positive colorectal cancer patients. Cancer Res 1997;57:2485–2492.

24 Van Cutsem E, Nowacki M, Lang I et al. Randomized phase III study of irinotecan and 5-FU/FA with or without cetuximab in the first-line treatment of patients with metastatic colorectal cancer (mCRC): The CRYSTAL trial. J Clin Oncol 2007;25(18 suppl):4000.

25 Hurwitz H, Fehrenbacher L, Novotny W et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 2004;350:2335–2342.

High Concordance of KRAS Status Between Primary Colorectal Tumors and Related Metastatic Sites: Implications for Clinical Practice

Daniele Santini, Fotios Loupakis, Bruno Vincenzi, Irene Floriani, Irene Stasi, Emanuele Canestrari, Eliana Rulli, Paolo Enrico Maltese, Francesca Andreoni, Gianluca Masi, Francesco Graziano, Giacomo Giulio Baldi, Lisa Salvatore, Antonio Russo, Giuseppe Perrone, Maria Rosa Tommasino, Mauro Magnani, Alfredo Falcone, Giuseppe Tonini and Annamaria Ruzzo

Oncologist 2008;13;1270-1275; originally published online Dec 4, 2008;
DOI: 10.1634/theoncologist.2008-0181