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Concordant Analysis of KRAS Status in Primary Colon Carcinoma and Matched Metastasis

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Abstract. *KRAS somatic mutations are the main predictive factor for non response to EGFR-targeted monoclonal antibodies in metastatic colorectal cancer (mCRC) patients. We compared KRAS mutational status in the primary tumour and the corresponding metastases (1 to 4 sites) in 38 mCRC patients. KRAS mutational status was analysed using direct sequencing, SNAPShot multiplex PCR and Scorpion Taqman PCR analysis. Results showed 54% of primary tumours had KRAS mutations. A concordance of 97% between primaries and metastatic sites was observed. A tumour heterogeneity was also demonstrated in 5% of mCRC. One case with three different primary tumours harboured three different KRAS mutations, and only one was represented in the unique metastasis of this patient. We concluded there was a high concordance in the KRAS status between the primary tumour and metastases. More than one informative block and more sensitive assay may increase the accuracy of KRAS status determination.*

Activating mutations of the *KRAS* gene family are the most common genetic events in tumourigenesis and have been shown to be highly predictive of the response to anti-epidermal growth factor receptor antibodies (anti-EGFR) in colorectal cancer (CRC). Lievre *et al.*, first reported the link between *KRAS* mutations and lack of response to EGFR-targeted monoclonal antibodies in patients with metastatic CRC (mCRC) (1). Then several studies confirmed that *KRAS* somatic mutations (codons 12 and 13) may predict lack of response to cetuximab- and panitunumab-based treatments in mCRC patients (2-9). These drugs have been approved as first, second and third line therapies for mCRCs (10-12). Therefore mutational analysis is mandatory before treatment, and reliable benchmarks for the frequency and types of *KRAS* mutations must be established to enable routine testing of mCRCs. These results have affected the way anti-EGFR drugs are prescribed; the European Medicine Agency (EMEA) has restricted drug prescription to patients with wild type (wt) *KRAS* in tumours because tumour with mutated *KRAS* did not respond to anti-EGFR antibodies.

Mutational analysis data have considerably improved the use of the anti-EGFR-targeted monoclonal antibodies in the treatment of colorectal cancer (1, 4, 5). However this method of targeting treatment to specific patients remains somewhat problematic. While the tests for lack of response to anti-EGFR antibodies is highly specific (nearly 95% of the patients with codons 12 or 13 mutations of *KRAS* gene failed to respond to therapy), it lacks sensitivity. Patients with wt *KRAS* gene failed to respond to anti-EGFR-targeted monoclonal antibodies in 40 to 60% cases (4). These data suggest that either the methods used are not sufficiently sensitive, or that other molecular determinants of response have yet to be identified. In addition, most studies have been conducted on samples from primary tumours, rather than metastases (1, 3), with only a few retrospective studies being conducted in primary tumours and their corresponding metastases (13).

There is growing evidence that the presence of *BRAF* mutations in colorectal tumours predicts non response to EGFR-targeted monoclonal antibodies (14-17). In the studies published to date, the frequency of *BRAF* mutation ranged from 3% to 17% and appeared exclusively with *KRAS* mutations. It has been suggested that *BRAF* V600E mutation status is a marker of poor prognosis in metastatic colorectal cancer and should be taken into account before considering EGFR-targeted monoclonal antibodies in colorectal cancer (14).

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Table I. *Patient clinical and pathological characteristics*.

Characteristic	number of patients (%)
Sex (female/male)	19/19 (50/50)
Median age (range)*	62.5 (23-89) years
Primary tumour site	
Proximal colon	12 (32)
Distal colon	15 (39)
Rectum	11 (29)
Metastases	
Synchronous	24 (63)
Metachronous	14 (37)
Sites of metastases	
Liver	37 (97)
Ovaries	5 (13)
Peritoneum	1 (3)
Others	3 (8)
Number of organs involved	
1	31 (82)
2	4 (11)
3	2 (5)
4	1 (3)

*At first treatment.

KRAS mutational status is assessed mainly in primary tumours whereas the treatment is administered in patients with mCRC. The distribution of *KRAS* mutations in primary and metastatic sites is clearly of interest. The aim of this study was to evaluate the degree of concordance between *KRAS* mutations in primary tumours and related metastases in order to investigate whether the *KRAS* status is stable in both synchronous and metachronous metastases. In order to improve the understanding of these results, the study also evaluated the degree of concordance between different sites of metastases or between different primaries. The mutational status of these genes between the primary tumour and the corresponding metastases was respectively compared for 38 patients by genomic sequencing, shot analysis and Taqman analysis.

Patients and Methods

Demographics of patients and tumour sample selection. Thirty eight patients treated for mCRC at the Institut Curie, Paris, France from January 1998 to January 2003 were included in this retrospective study. They consisted of 19 women (50%) and 19 men (50%). The patient's average age at primary diagnosis of metastasis was 65±12 years. Two patients had stage I tumour, 1 patient had stage II tumour, 3 patients had stage III tumour and 32 patients had stage IV tumours at initial diagnosis. The other clinical characteristics of the patients are reported in Table I. Tumour samples were taken in a routine diagnostic analysis of *KRAS* assessment from primary tumours and metastases for decision regarding the use of EGFR-targeted monoclonal antibodies for treatment.

Table II. Primers and polymerase chain reaction (PCR) conditions for mutational analysis.

Mutations	Primers	PCR conditions
KRAS codons 12-13	F-5'-GTATTAACCT TATGTGTGACA-3' R-5'-GTCCTGCACC AGTAATATGC-3'	58˚C 30 s
BRAF codon 600	F-5'-TGCTTGCTCTG ATAGGAAAATG-3' R-5'-AGCATCTCAG GGCCAAAAAT-3'	58˚C 30 s

Cases that did not have sufficient quantities of surgical or cytological material available for testing were excluded. All were subjected to analysis on the metastatic and primary tumour material. *DNA extraction*. Genomic DNA was extracted from three 15 µm-thick AFA-fixed paraffin-embedded tissue sections from primary and metastatic lesions by proteinase K digestion and the Qiamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The percentage of tumour cells present in the sample was evaluated on a haematoxylin-eosin-stained section of a representative block for each sample by microscopic examination by a pathologist. Macro-dissection of the invasive or metastatic carcinoma excluding normal colon, hepatic tissue, adipose tissue, muscular tissue inflammatory cells was performed to increase the cellularity when below 60% (range 5 to 59%) in 48 out of 119 tissue samples studied (40%).

Mutational analysis of KRAS and BRAF by direct sequencing. All samples were screened for *KRAS* codon 12 and 13 mutations and *BRAF* V600E mutation. The analysis was performed using DNA amplification followed by direct sequencing. Primer sequences and cycling conditions are shown in Table II. Each PCR reaction contained 50-250 ng of genomic DNA, 0.4 µM of each primer, 12.5 µl of PCR master mix and 0.625 U of Hotstart Taq polymerase (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl. Successful and specific amplification was verified by visualizing 10 µl of the PCR product on a 2% agarose gel. All samples were subjected to automated sequencing by ABI PRISM 3130 using the BigDye Terminator kit (Applied Biosystems). All samples were analysed twice, starting from independent polymerase chain reactions. Forward and reverse sequences were analysed using Seqscape v. 2.5 software (Applied Biosystems). Each case was classified as positive or negative for the *KRAS* and *BRAF* mutation based on the comparison of the sequence to the wild-type sequence.

KRAS detection by SNAPshot multiplex assay. PCR-amplified KRAS exon 2 was purified then analysed for the presence of mutations at nucleotides c34, c35, c37 and c38 using the ABI PRISM SNAPshot Multiplex kit (Applied Biosystems) and four primers including at their 5' end and additional tail allowing their simultaneous detection as previously described [3].

Labelled products were separated using an ABI PRISM 3130 DNA sequencer and data were analysed using Peak Scanner Sofware (Applied Biosystems).

Table III. *KRAS mutations in primary tumour and metastatic sites.*

Patient no.	Gender	Primary tumours			Metastatic sites			Concordance (yes=1/no=0)
		Seq.	SNAPshot	Scorpion Taqman	Seq.	SNAPshot	Scorpion Taqman	
1	F	wt	wt	wt	wt	wt	wt	1
2	M	G12C	G12C	G12C	G12C	G12C	G12C	1
3	F	G12V	G12V	G12V	G12V	G12V	G12V	1
4	M	wt	wt	wt	wt	wt	wt	1
5	M	wt	wt	wt	wt	wt	wt	1
6	F	NI	wt	wt	wt	wt	wt	1
7	F	G13D	G13D	G13D	G13D	G13D	G13D	1
8	F	G12D	G12D	G12D	G12D	G12D	G12D	1
9	M	G12D	G12D	G12D	G12D	G12D	G12D	1
10	M	wt*	wt*	wt*	wt	wt	wt	1
11	F	G13D	G13D	G13D	wt	wt	wt	0
12	F	G13D	G13D	G13D	NI	G13D	G13D	1
13	M	NI	wt	wt*	wt	wt	wt	1
14	M	wt	wt	wt	wt	NI	wt	1
15	M	wt	wt	wt	wt	wt	wt	1
16	F	G12D	G12D	G12D	G12D	G12D	G12D	1
17	F	NI	G13D	G13D	G13D	G13D	G13D	1
18	M	G12V	G12V	G12V	NI	G12V	G12V	1
19	F	NI	wt	wt	wt	wt	wt	1
20	M	wt	wt	wt	wt	wt	wt	1
21	F	wt	wt	wt	wt	wt	wt	1
22	F	wt	wt	wt	wt	wt	wt	1
23	F	G12D	G12D	G12D	G12D	NI	G12D	1
24	M	G13D	G13D	G13D	G13D	G13D	G13D	1
25	F	wt	wt	wt	wt	wt	wt	1
26	M	G12D**	G12D**	G12D**	G12D	G12D	G12D	1
27	F	G12D	G12D	G12D	G12D	G12D	G12D	1
28	M	G12D	G12D	G12D	G12D	G12D	G12D	1
29	M	G13D	G13D	G13D	G13D	G13D	G13D	1
30	M	G13D	G13D	G13D	G13D	G13D	G13D	1
31	F	G12S	G12S	G12S	G12S	G12S	G12S	1
32	M	NI	wt	wt	wt	wt	wt	1
33	F	G13D	G13D	G13D	G13D	G13D	G13D	1
34	M	wt	wt	wt	wt	wt	wt	1
35	F	wt	wt	wt	wt	wt	wt	1
36	F	wt	wt	wt	wt	wt	wt	1
37	M	wt	wt	wt	wt	wt	wt	1
38	M	G12V	G12V	G12V	G12V	G12V	G12V	1

*Three different adjacent primary tumours: G12V, wt, G12A one metastasis wt, **three slides of the same block wt, wt, G12D, one other block kG12D; NI: not identified.

KRAS detection by allele-specific real-time PCR (ARMS®) combined with Scorpions® real-time PCR. Mutant KRAS was also determined using a kit identifying seven somatic mutations in codons 12 and 13 (Therascreen™ DxS Ldt, Roche, Manchester, UK). Allele specific real-time quantitative PCR was carried out on ABI 7500 HT Sequence detection System (Applied Biosystems) according to manufacturer's instructions. The kit detects 7 KRAS mutations in codons 12 and 13 (G12D, G12A, G12V, G12S, G12R, G12C and G13D)

Quality assessment of results. Samples with mutated status were analysed regardless of the percentage of tumour cells present. *KRAS* status was determined taking into account the results of the analysis of independent triplicate experiments. When the mutational analysis revealed non interpretable results, additional tumour samples from the primary tumours or the distant metastases were collected and analysed. When poor quality of DNA did not allow a conclusion to be drown, with one of the assays (results non interpretable, NI) but give interpretable and concordant results with the two others assays, we used these latter results.

Data analysis. A comparison of mutational status between primary tumours and metastatic sample(s) was undertaken for each patient to define the concordance of the *KRAS* mutational status between the tumour types. Sensitivity of each assay was determined by the

Table IV. *Detailed KRAS mutations in primary tumour and different metastatic tumours of the same patient.*

Patient no.	Primary tumours (PT)	Metastatic sites (M)				
	Liver	Ovaries	Peritoneum	Lung	Douglas	Lymph nodes
12	G13D	G13D	G13D	G13D	G13D	
16	G12D	G12D	G12D			
27	G12D	G12D				G12D
29	G13D	G13D			G13D	
31	G12S	G12S	G12S	G12S		G12S
32	wt	wt		wt		

Table V. *Detailed KRAS mutations in primary tumour and metastatic in patients with more than one round analysis in different blocks of the same tumour.*

Patient no.	Primary tumours (PT)			Metastatic sites (M)	
	PT1	PT2	PT3	M1	M2
4	wt	wt		wt	
5	wt	wt		wt	
6	wt	wt		wt	
7	wt	G13D	G13D	G13D	G13D
12	G13D	G13D		G13D	G13D
13	wt	wt		wt	
15	wt	wt		wt	
16	G12D	G12D		G12D	
19	wt			wt	wt
23	G12D	G12D		G12D	
24	G13D	G13D		G13D	G13D
26	wt	G12D	G12D	G12D	
31	G12S	G12S		G12S	

percentage of concordant results in the three methods used (direct sequencing, SNAPShot multiplex PCR and Scorpion Taqman PCR analysis for each sample).

Results

Thirty-eight cases of colorectal cancer and matched metastases were analysed for *KRAS* and *BRAF* mutations. The histological subtype of the primary tumour was invasive adenocarcinoma including 12 well-differentiated tumours (33%), 20 moderately (56%) and 4 poorly differentiated carcinomas (11 %) and for 2 samples, the differentiation status was not available (5%).

Tumours from all 38 patients had sufficient material in the primary and the metastatic sample to be analysed. All samples were available for *KRAS* mutational analysis and comprised more than 60% tumour cells when the *KRAS* status was wt.

Results of *KRAS* status were presented in Table III. Primary tumours from 20 (54 %) patients contained *KRAS* mutations (8 G13D, 7 G12D, 3 G12V, 1 G12S and 1 G12C). Primary tumours from 18 patients had a wt status (46 %). Metastatic tumours from 19 patients (50%) contained *KRAS* mutations (7 G12D, 7 G13D, 3 G12V, 1 G12S and 1 G12C). Metastatic tumours from 19 patients had a wt status (50%). For 1 patient (3% of all cases) (pt # 11, Table III) inconsistent results were observed for *KRAS* mutations. For this patient, the G13D mutation was restricted to the primary tumour, but was not found in the distant metastases. Only a biopsy of a single metastatic site was available. Three different AFA-fixed tumour blocks of the primary tumour were analysed, all harbouring G13D mutation.

No V600E *BRAF* mutation was observed in any of the patients, either in wt tumours or in *KRAS*-mutated samples. No relationship was observed between *KRAS* mutational status and the degree of differentiation of the tumour or the stage of the tumour.

The initial biopsy and the corresponding tumour obtained for the diagnosis at surgery was analysed for 6 patients. All samples were also concordant either on the primary tumour for 5 samples: 1 sample wt (pt #22), 1 G12V (pt #3), 2 G12D (pts #8, # 9) 1 G13D (pt #29) or on the metastases (1 sample wt, pt #20).

Different metastatic sites (2 to 4 sites) were analysed for 6 patients: 5 liver metastases (pts #12, #16; #27; #29; #31), 3 ovarian metastases (pts #12; #16; #31), 2 metastases of the peritoneum (pts #12, #16, #31), 2 lung metastasis (pt #29), 1 Douglas metastasis (pt #12, #31), 2 metastatic lymph nodes (pts #27; #31) (Table IV). The same distant metastatic site was analysed at different times during disease follow up (liver metastases, 3 cases: pts #7; #19; #20). The same *KRAS* status (G13D, wt and wt, respectively) was observed. The distribution of mutational status was not related to the metastatic site, all mutations were represented whatever was the site of the mutation.

One patient (pt #10, Table III) presented 3 synchronous distinct but adjacent primary tumours on the distal colon, one well-differentiated adenocarcinoma and 2 poorly differentiated adenocarcinomas. The *KRAS* status was different in each tumour, one G12V (well-differentiated tumour), one wt and one G12A (the 2 poorly differentiated tumours). The patient presented diffuse liver metastasis; a biopsy of only one metastasis was performed. The *KRAS* status was wt, concordant with the second tumour (poorly differentiated).

For 25 of the patients, only one block of the primary and metastatic tumour was analysed. For 13 of the patients different blocks of primary and/or metastatic site (2 or 3 different blocks) were analysed (Table V). Results were

concordant for all cases, except for 2 patients (pts #7 and #26). For patient #7, 3 different tissues block of the primary tumour were analysed. In one block, wt *KRAS* status was observed, whereas in the two other blocks G13D status was observed. The same G13D *KRAS* mutation was observed in two blocks of the corresponding liver metastasis. For patient #26, 3 different tissues block of the primary tumour were analysed. In one block, wt *KRAS* status was observed whereas in the two other blocks G12D status was observed. The same G12D *KRAS* mutation was observed in the liver metastasis.

The comparison of the sensitivity of the three methods of analysis demonstrated that 100% of the mutations were detected using the Therascreen DxS kit. None of the primary tumours but 2 metastatic samples were not interpretable using the SNAPshot assay (2/76 samples; 3%) and 5 primary and 2 metastatic samples (7/76 samples; 9%) were not interpretable by direct sequencing (Table III).

Discussion

Although the EGFR protein is expressed in approximately 85% of metastatic colorectal cancer tumours (18), only a subset of patients will have a clinical benefit from treatment with EGFR-targeted monoclonal antibodies. There is now strong evidence that the efficacy of these drugs is limited to patients whose tumours carry a wt *KRAS* gene (1-3, 5, 6, 16). Activating mutations of *KRAS* which could result in EGFR-independent intracellular signal transduction activation are almost exclusively detected in codons 12 and 13 of exon 2 *KRAS* gene. Mutations in codons 61, 146 or 154 have also been described in 1% of colorectal cancer (19). Mutations in codon 12 are the most frequent (80%) (20, 21). Mutations in *BRAF*, that encodes a serine/threonine kinase that function downstream KRAS, occurs in 3-17% of colorectal cancer and are usually exclusive with a *KRAS* mutation (22, 23).

In the current series, half of the patients had *KRAS* mutation, representing a higher percentage than in the overall population of patients with colon cancer (average 30 to 40%) (14, 24). This is probably related to the bias of representation of the current series of patients with 32 of the 38 patients (84%) having stage IV disease.

Molecular testing in colorectal cancer is usually performed on formol or AFA-fixed samples. The main factors that will impact on assay design in solid tumour testing are tumour purity, type fixation processing of the tissues and tumour (DNA) quantity. Tumour purity is essential because false negative results will be common when the sample is contaminated at a high level with non tumourous cells. Macro-dissection is frequently needed to enhance the tumour cellularity of the sample. For wt samples, a minimum of 50% of tumour cells are needed in order to obtain an accurate result. However, many laboratories require a sample to contain at least 75% tumour cells for testing accuracy (25). The type of fixative used and the duration of fixation are also important factors. Picric acid containing solutions, leading to DNA degradation, generally prohibit molecular testing. In addition, tissues fixed for more than 24 hours have a much lower yield and poorer DNA quality, which can result in failed molecular testing (26).

Different molecular assays are used for detecting *KRAS* point mutations in tumours. They include dideoxy sequencing, which is the traditional cycle sequencing reaction on the basis of the Sanger method of gene sequencing, and allele specific PCR (3, 9). These assays have been largely used in clinical trials. This study used *KRAS* sequencing, since it is considered the gold standard for detection of mutations. Sequencing is a highly specific technique, with a very low false-positive rate. The false-positive rate is here reduced by performing duplicate sequencing in both forward and the reverse directions and by independent duplicate analysis with another technique. The major pitfall of direct sequencing is that it is not very sensitive. The analytic sensitivity of this method is reported to be 10-30% of mutant *KRAS* in a background of wt sequence (27). It generally requires 20-25% mutant cells to be detected. In addition it requires sufficiently high quality material to decrease the background. SNAPshot methods based on multiplex reaction have been reported as a specific and more sensitive assay than sequencing (3). Real-time based assays are also an attractive option for clinical testing because they are rapid and have a better sensitivity than direct sequencing. This study used a commercially available assay (Therascreen™ KRAS kit, DxS, Manchester, UK) that is an allele-specific assay, using multiple probes that are specific for each one of the most common 7 mutations described in codons 12 and 13. The relative sensitivity of these methods is confirmed by the current study where 100% samples were analysable by QPCR analysis, whereas 96% samples were analysable by SNAPshot method and 91% by direct sequencing. Interestingly, the specificity of these three methods is high. This is representative of the reported data and different assays. During the past decade, improvement of molecular technology has allowed the sensitivity and the specificity of the mutation detection to be improved, rendering it useful in the clinical setting (24, 28, 29).

Most published analyses have been conducted on samples from primary tumours. Few data have been reported in primary sites and their corresponding metastases. This study demonstrated a good correlation between primary and metastatic tumour *KRAS* status (97% concordance). Two previous studies also reported a high concordance of *KRAS* status between primary colorectal tumours and related metastatic sites (13, 30, 31). One study was conducted in 99 colorectal carcinomas and metastasis, and included 80% liver metastasis, 7% lung metastases, and 12% other metastatic sites (13). *KRAS* status was analysed by direct sequencing. The authors showed 96% concordance between primaries and metastatic sites. The second retrospective study was performed

in 124 patients and 138 related metastatic sites (30, 31). The sites of metastases were liver in 83% of the cases, lung in 16.7% of the cases. *KRAS* status was also analysed by direct sequencing. Ninety three percent concordance was observed (30, 31). A recent publication highlighted the possible tumour heterogeneity, and as in the present study, the authors analysed different parts of each tumour: tumour centre and invasion fronts (32). In that study, a higher rate of *KRAS* mutations was detected in the tumour centre compared with the invasion front. The authors suggested that tumour samples should preferably be taken in the tumour centre (32). A similar heterogeneity in 2 patient samples in the current study led the authors to choose to perform central punch biopsy sampling in prospective routine analysis. Another older study showed heterogeneity between primary colorectal carcinomas and matched metastases (33). This study included 30 matched samples and demonstrated more *KRAS* mutations in primaries than in metastatic sites (14 *vs*. 13) and 9 discordant cases (33). However, *KRAS* status was analysed by single-strand conformation polymorphism SSCP analysis of DNA molecules amplified from the first exon of *KRAS* and it can therefore be hypothesized that the sensitivity of this assay is lower than recent quantitative PCR assays.

Interestingly, in this series, one patient had synchronous primary tumours with 3 different mutational statuses (WT, G12V and G12A). Only one representative concordant status was found in the unique metastatic site. Similar data were previously published for colon adenomas (34). In this paper, one patient had a G12D mutation in invasive primary colon tumour and the corresponding metastasis. However, the same patient also had 3 colon adenomas with 3 different mutations: one G12D, one G12C and one G12V. In the same paper, the authors reported another patient with 3 specimens of primary carcinoma and 2 lymph node metastases with a G12V *KRAS* mutation, but only one of the two corresponding liver metastasis had a G12V mutation (34). The detection of *KRAS* mutations was performed by allele-specific oligonucleotides hybridization to PCR-amplified DNA from microdissected tissue. Our results and these data emphasize the interest of multiple site analysis in order to determine the actual utility of anti-EGFR antibodies for the patient.

The current analysis confirms the high concordance level of determination of *KRAS* status on the primary colorectal tumour and its related metastases. However, this study also highlighted the role of a high content of tumour cells of the sample (macrodissection or punch biopsies) and the use of duplicate independent assays to increase the accuracy of the test. The heterogeneity of the tumour and the possible difference in *KRAS* status in the synchronous primary tumour or metastasis emphasizes the role of multiple sampling to improve the accuracy and the sensitivity of the *KRAS* status determination.

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