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SHORT COMMUNICATION

Acquired *KRAS* mutations during progression of colorectal cancer metastases: possible implications for therapy and prognosis

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Abstract
Purpose Documentation of a wild-type (*wt*) *KRAS* gene in tumor has become mandatory for the prescription of anti-EGFR monoclonal antibodies in patients with colorectal cancer (CRC). Acquired *KRAS* mutations have seldom been reported in metastases from *wt KRAS* primary CRC. We report the first case of multiple *KRAS* mutations acquired during the metastatic phase of CRC, and retrospectively reviewed all patients with CRC, in whom *KRAS* was analyzed in at least two tumor samples from distinct lesions.
Methods Genomic DNA purified from paraffin-embedded tissues was used after histological quantification of tumor tissue. The seven *KRAS* mutations located within codons 12 and 13 were screened using the allelic discrimination assay.
Results A 35-year-old woman with CRC liver metastasis, resistant to all conventional cytotoxic agents, experienced for the first time significant tumor shrinkage while cetuximab was added, allowing hepatic resection. Further liver relapse occurred on cetuximab, but a new hepatic resection was attempted. No mutation in *KRAS* was detected in the primary colon tumor or in synchronous liver metastases. In contrast, in metachronous liver metastasis samples, two distinct mutations at codon 13 and 12 were detected. No acquired mutations were found in all the other 12 CRC cases with at least two serially performed *KRAS* analyses.
Conclusions Our findings suggest that late switch in *KRAS* mutational status could occur more frequently than currently recognized and account for acquired resistance to anti-EGFR therapies. Prospective studies are warranted to better estimate the incidence of change in *KRAS* mutational status and assess their clinical relevance.

Keywords *KRAS* mutation · Acquired · Metastases · Colorectal cancer

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Introduction

Mutations of the *KRAS* gene have been recently shown to predict resistance to epidermal growth factor receptor (EGFR)-targeted monoclonal antibodies [1–3]. *KRAS* wild type (*wt*) is now a standard requirement for prescription of

anti-EGFR therapy in patients with metastatic colorectal cancer (mCRC) [4, 5]. Nevertheless, little is known about the evolution of the *KRAS* mutational status and subsequent gene mutations during the spontaneous course of disease progression and on chemotherapy. In particular, it is unclear whether *KRAS* mutations are always early events, or could be acquired at later stages of CRC. We report the case of a patient with mCRC, in whom tumor *KRAS* mutations were acquired after the occurrence of the first metastasis.

Case report

A 35-year-old-female patient, without personal or familial history of cancer, was diagnosed with adenocarcinoma of the transverse colon and synchronous bulky liver metastases. Immunohistochemistry for MLH1, MSH2 and MSH6 on tumor and healthy liver samples revealed no evidence for hereditary non-polyposis colon cancer (HNPCC). She received 8 cycles of a fluorouracil (5FU) and oxaliplatin-based chronomodulated regimen, which achieved disease stabilization. Irinotecan was added to the regimen for seven cycles, but the patient demonstrated only minor tumor response. A segmental transverse colectomy was then performed, followed by eight cycles of the same triple therapy, but with alternating intravenous (IV) and hepatic arterial infusions (HAI), without any tumor regression. Intravenous cetuximab was therefore added to the previous alternating regimen over 12 additional cycles. This regimen achieved a partial tumor response, associated with grade 2 acneiform rash. The patient could then undergo left hepatectomy and segment VII liver resection. Pathologic examination of the specimen demonstrated viable hepatic metastases with positive surgical margins (R1 resection). Six post-operative cycles of HAI, followed by IV triple therapy associated with IV cetuximab were administered. In spite of continued therapy, new lesions subsequently developed in the liver and the peritoneum. The addition of bevacizumab to the previous regimen did not halt disease progression. Nevertheless, the limited size and number of the progressive lesions prompted a segment V hepatectomy and the resection of both peritoneal nodules. This surgical procedure achieved complete clearance of all macroscopic disease 11 months after the first hepatectomy. The patient received eight post-operative cycles of IV cetuximab and irinotecan/5FU for an additional 6 months with no evidence of disease. Two months after chemotherapy discontinuation, new hepatic and peritoneal lesions were discovered. The patient received various palliative irinotecan, fluoropyrimidine, oxaliplatin, cetuximab, bevacizumab-based regimens resulting at best in stable disease, and finally was lost to follow-up in a clinical setting of tumor progression and general status deterioration in July 2008.

Methods

A retrospective analysis of the *KRAS* mutational status was performed on tissues obtained from the primary tumor and subsequently resected liver metastases. Genomic DNA purified from paraffin-embedded tissues was used after histological quantification of tumor tissue in each tumor sample by hematoxylin–eosin–saffron (HES) coloration. The percentage of viable tumor in all the samples analyzed for *KRAS* mutation varied from 30 to 55% (Table 1). The seven *KRAS* mutations located within codons 12 and 13 were screened using an allelic discrimination assay using primers and specific probes for each mutated and non-mutated allele as previously described [6]. Briefly, reactions were performed in 15 µl comprising 20 ng of DNA, 1× of specific primers and probes, and 1× Taqman Genotyping Master Mix (Applied Biosystems, Foster City, CA). DNA was then submitted to PCR cycle conditions on, and analyzed with a Lightcycler 480 instrument (Roche Applied Science, Mannheim, Germany). The analysis of each sample was performed in duplicate, and DNA from wild-type sample and cell lines exhibiting each *KRAS* mutations were used as controls in each experiment. The detection threshold of our technique was tested using dilution of DNA bearing the various *KRAS* mutation into normal DNA using the same methods as for patient samples. All mutations were detectable

Table 1 Case report: *KRAS* mutational status in primary tumor and liver metastases

Type and date of surgery	Sample origin	Proportion of tumor cells (%)	*KRAS* status
Segmental colectomy OCT, 2004	Primary colon	40	*wt*
First hepatectomy AUG 2005	Synchronous liver metastasis (lesion # 1)	30	*wt*
	Synchronous liver metastasis (lesion # 2)	55	*wt*
Second hepatectomy JUL 2006	Metachronous liver metastasis (lesion # 3)	50	Mutated G38A/G13D
	Metachronous liver metastasis (lesion # 4)	40	Mutated G38A/G12D

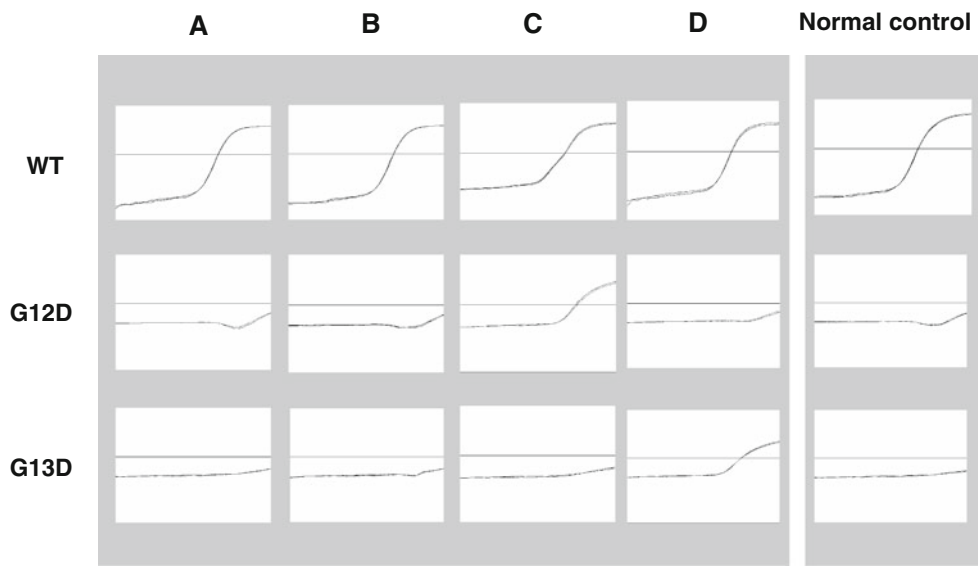

Fig. 1 Mutation analysis of the *KRAS* gene in the reported patient. Amplification curves using allelic discrimination assay are shown for the primary colon tumor sample (**a**), one synchronous liver metastasis (**b**) and two metachronous liver metastatic nodules from the second hepatectomy (**c** and **d**). *First, second and third rows* correspond to the amplification curves obtained with the wild-type, G12D and G13D specific probes, respectively

up to dilution of 1%, except G12V up to 5% and G12S up to 10%. Each sample analysis was performed in duplicate, and wild-type and mutated *KRAS* controls using DNA extracted from cell lines with known *KRAS* mutational status were used in each experiment. As shown in Table 1 and Fig. 1, no mutation in *KRAS* was detected in the primary colon tumor or in synchronous liver metastasis samples. In contrast, in the metachronous liver metastasis from the second hepatectomy, mutations at codon 13 and 12 were detected in two separate nodules.

We then retrospectively reviewed all other patients with CRC, in whom *KRAS* was analyzed, using the same technique, in at least two tumor samples from distinct lesions. Thirty-three separate analyses were retrieved from 25 tumor sites in 12 patients (Table 2). A *KRAS* mutation was found in eight of nine tumor sites collected in four patients. Three patients with mutated tumor had the same codon 12 mutation in all collected samples (Table 2; # 1–3). One patient with a bifocal primary colon tumor had a mutation at codon 13 in only one of them, no mutation in a synchronous liver metastasis and the same mutation in one of two specimens of a subsequent locoregional relapse (Table 2; # 4). No acquired mutations were detected in any other patient, beside the current case report. However, only 5 of the 12 patients had *KRAS* analyzed in metastatic or locoregional recurrences occurring sequentially during disease history, comparable to our case report.

Discussion

Cancer progression is characterized by genomic instability and accumulation of somatic mutations. It is therefore not surprising to observe the development of new mutations during the course of metastatic spread. Nevertheless, *KRAS* mutations were initially reported as very early events in colorectal carcinogenesis. Even before malignant transformation, about 30–35% of benign colorectal adenomas bear a *KRAS* mutation, a proportion similar to that observed in invasive cancer [7, 8]. A similar *KRAS* mutational status was found both in primary tumor and in metastases for more than 90% of the patients with CRC [9–12] or lung cancer [13, 14]. This finding is consistent with *KRAS* mutations mostly occurring as an early molecular event. However, both studies also document a few cases of *KRAS* mutations in metastases arising from *wt KRAS* primary tumors. To the best of our knowledge, we report for the first time a change in *KRAS* mutational status in two sequential samples of metastatic lesions in the same organ during the course of disease progression.

Evolution of *KRAS* mutations in this setting might be secondary to clonal selection of cells with early mutated *KRAS* in a given tumor under pressure from anti-EGFR therapy. This selection would render a previously cetuximab sensitive tumor unresponsive. Alternatively, a novel spontaneous mutation in cancer cells could explain the finding of *KRAS* mutations in metastases, where previously none existed. It is unknown whether exposure to cytotoxic chemotherapy favors *KRAS* mutation. Neither is it known whether acquired *KRAS* mutations are observed more frequently in patients with germ-line deficit in DNA repair systems, such as HNPCC patients. In our patient with an early CRC onset, HNPCC was ruled out, yet no other DNA repair deficiency was sought.

The methodology in this report utilizes a widely accepted technique [5, 6]. The proportion of tumor tissue was appropriate for the technique used to detect gene mutations [4, 5] in our case report (Table 1), while, among the

Table 2 *KRAS* mutational status in 12 other patients with mCRC and *KRAS* analysis in tumor samples collected sequentially

Nb	Gender	Primary	Biopsy sites	Interval between biopsies (months)	Occurrence of lesions	% tumor tissue in the analyzed sample	Mutation type
1	M	Rectum	Primary Liver metachronous	10	Sequential	25 40	G12D G12D
2	M	Rectum	Liver metachronous Liver metachronous	2.5	Simultaneous	70 20	G12C G12C
3	M	Rectum	Liver synchronous Liver synchronous Liver synchronous	2.5–4.5	Simultaneous	15 20 50	G12D G12D G12D
4	F	Sigma and right colon (bifocal)	Liver synchronous Primary (right colon)	4	Simultaneous	20 40	wt G13D
5	M	Left colon	Liver synchronous Liver metachronous	24	Sequential	40 55	wt wt
6	M	Sigma colon	Primary Pelvic recurrence	33	Sequential	60 10–40	wt wt
7	M	Lower rectum	Liver metachronous Liver metachronous	15	Sequential	50 40	wt wt
8	M	Sigma colon	Liver metachronous Lymphadenopathy	28	Sequential	35 40	wt wt
9	M	Sigma colon	Liver synchronous Liver synchronous	11	Simultaneous	15 60	wt wt
10	M	Sigma colon	Lung metachronous Lung metachronous	3	Simultaneous	50	wt wt
11	M	Left colon	Liver synchronous Liver synchronous	4	Simultaneous	20 90	wt wt
12	M	Right colon	Primary Liver synchronous	4	Simultaneous	Not available	wt wt

other cases tested, some negative samples had an unknown or insufficient percentage of tumor cells and may be unreliable (Table 2, # 6, 9, 12). Furthermore, the sensitivity of our test, measured by a DNA dilution technique, was even higher than that (20%) reported by Lievre et al. [6]. This finding may be due to technical differences introduced when adapting the method to our local specificity. In particular, PCR master mix and PCR instrument were different than those described in the original publication [6]. Furthermore, all specimens were independently tested in duplicate. It is therefore unlikely that the finding of a late *KRAS* mutation would be explained by a false negative result in the samples from the primary tumor and the first hepatectomy. In addition, the clinical course of this patient is consistent with the late change in *KRAS* status. Albeit heavily exposed to all active drugs used to treat mCRC, only the initial introduction of cetuximab resulted in clinically relevant tumor shrinkage, while the tumor was harboring a *wt KRAS* gene. Subsequently, the tumor progressed on cetuximab therapy, and tumor tissue from the second hepatectomy showed a mutated *KRAS* gene. Of note, two different mutations were detected in two separate histological samples, suggesting multiple mutations, or secondary selection of multiple cetuximab-resistant clones during cetuximab therapy.

Intraneoplastic heterogeneity of cancer populations is a well-known phenomenon that could determine the genesis of potential drug-resistant metastatic clones [15–19] Similarly, tumor heterogeneity is a limit of tumor biomarker analyses [20], illustrated in our case by the presence of two distinct mutations in two nodules sampled during the same metastatic phase. The lack of *KRAS* mutations in the initial samples could have been due to sampling in a non-mutated area of the tumor [19]. It is likely that increasing the number of samples analyzed in one tumor location would increase the incidence of discrepant *KRAS* status. The limits of the conventional technique using DNA extracted from formalin-fixed tumor samples could be also improved by more sensitive techniques and other DNA source [21].

Finally, assessment of *KRAS* status for therapeutic purposes represents a new paradigm in cancer therapy. Our findings suggest that a late switch in *KRAS* mutational status could occur more frequently than currently recognized and account for acquired resistance to anti-EGFR therapies. Progressive metastases unresponsive to treatment do not benefit from metastasis resection [22] and are usually not resected. *KRAS* analysis is therefore not available at the time of progression. In this respect, our patient was an

exception as she was operated despite tumor progression, due to limited tumor location and size. Of note, *wt KRAS* metastases can be seen despite mutated *KRAS* in primary tumors and vice versa [9, 10]. This observation could justify serial assessments of *KRAS* mutations during the course of CRC in order to adjust therapeutic decisions and treatment strategies, especially in patients with tumor initially bearing *wt KRAS*. Prospective studies will be necessary to better estimate the incidence of change in *KRAS* mutational status through the course of metastatic disease and assess their clinical relevance.

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