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Simultaneous KRAS double mutation in codons 12-13 in a patient with colorectal cancer: a rare case-report

Antonio Taddei[1], Maria Novella Ringressi[1], Damiano Bisogni[1], Duccio Rossi Degl'Innocenti[2], Camilla Eva Comin[2], Paolo Bechi[1], Francesca Castiglione[2]

[1] Second Unit of General and Emergency Surgery, Careggi Teaching Hospital, Florence, Italy; [2] Department of Experimental and Clinical Medicine, Section of Surgery, Histopathology and Molecular Pathology, Careggi Teaching Hospital, Florence, Italy

Summary. Evaluation of the KRAS mutational status is a crucial step for the correct therapeutic approach in advanced colorectal cancer. According to well-established criteria, a molecular analysis of exon 2 (codons 12 and 13) is routinely performed in formalin-fixed, paraffin-embedded (FFPE) tissue and identification of a wild-type (WT) KRAS tumor may lead to more tumor-specific and less toxic treatment for the patient. The present study reports an additional case of the coexistence of two somatic mutations p.G12S (GGT>AGT) and p.G13D (GGC>GAC), in exon 2 of the KRAS gene, in the same selected tumor area in the same codon (codon 12) of exon 2 of the KRAS gene (uno dei due, non tutti e due) in a female patient suffering from an advanced adenocarcinoma of the rectum and hepatic metastasis, thus demonstrating the existence of intratumoral heterogeneity. Based on data in the literature, multiple mutations in the KRAS gene are infrequent, representing 2.1% of mutations in colorectal cancer, but their clinical significance is still unclear. The present study underlines the importance of intratumoral heterogeneity, as supported by the current data in which tumors may be polyclonal with a mixture of cell populations harboring varying mutations. In particular, the present case appears to support the hypothesis that the presence of multiple mutations in codon 12 is associated with a more aggressive disease not responding to chemotherapy.

Key words: KRAS simultaneous double mutation, colorectal cancer, codons 12-13

Introduction

Evaluation of the KRAS mutational status is a crucial step for the correct therapeutic approach in advanced colorectal cancer. According to well-established criteria, a molecular analysis of exon 2 (codons 12 and 13) is routinely performed in formalin-fixed, paraffin-embedded (FFPE) tissue and identification of a wild-type (WT) KRAS tumor may lead to more tumor-specific and less toxic treatment for the patient.

Numerous studies have demonstrated significant intratumoral heterogeneity, with spatially separated heterogeneous somatic mutations and chromosomal imbalances (1). With regard to colorectal cancer, several studies have highlighted the differences in the KRAS mutational status between primary and metastatic tumors within lymph nodes and visceral metastases or even different portions of the primary lesion (2), thus supporting the overall current theory of neoplastic heterogeneity (1, 3). However, the possibility of two or more mutations in the same codon of the

KRAS gene has seldom been reported in colorectal cancer and the real clinical impact of multiple mutations on patient prognosis has not yet been well studied or clarified (4-7).

The present study reports an additional case of the coexistence of two somatic mutations (p.G12S and p.G13D) in the same codon (codon 12) of exon 2 of the KRAS gene in a female patient suffering from advanced adenocarcinoma of the rectum and hepatic metastasis. This supports the possibility that there may be two clonal origins of the tumor along with concomitantly different mutations at the same genetic level.

Case report

In April 2005 a 64 year-old female patient (GM) was admitted to the AOU Careggi Hospital (Florence, Italy) due to diffuse abdominal pain and progressive constipation. After a clinical evaluation and radiologic examination suggesting an intestinal occlusion, she underwent a colonoscopy that showed an occludent hyperemic lesion of the splenic flexure. A colonic resection was performed and the histologic examination revealed a pT3 adenocarcinoma without metastasis in the 29 resected lymphnodes (pT3N0M0). Adjuvant chemotherapy consisting of 1,650 mg/m^2 capecitabina orally twice daily followed 4 weeks after surgery.

In February 2008, an increase in CEA serum levels was demonstrated (7.6 ng/mL) and a CT scan revealed a 6 cm, single, liver metastasis localized in the IV-VIII segment. She underwent an atypical liver resection and the subsequent histologic evaluation demonstrated a colonic adenocarcinoma liver metastasis. The patient began adjuvant chemotherapy on a CAPIRI regimen (Irinotecan 180mg/m^2 and Capecitabina 1,650 mg/m^2) and Avastin only in the last 3 cycles.

In March 2010 a follow-up CT examination revealed a new liver relapse in the IV segment. The patient began chemotherapy on a 6-month FOLFOX regimen (Oxaliplatin was administered on day 1 at the dose of 85 mg/m^2 as a 2 h infusion, concurrently with Leucovorin 200 mg/m^2/day, followed by bolus 5-Fluouracil (5-FU) 400 mg/m^2 and a 22 h infusion of 5-FU 600 mg/m^2 for two consecutive days), crossed with stereotaxic radiotherapy (RT) and Cyberknife treatment in November 2010, though without complete relief. In March 2012 a new relapse was demonstrated by CT scan and RT treatment was again performed. In February 2013 another progression was revealed and the patient began chemotherapy (FOLFOX). In October 2015 the patient is still alive and treatment is ongoing.

Materials and methods

Prior to the mutation analysis, the patient provided informed written consent. A tissue sample from the primary tumor was obtained from the archives of ASOD Istologia Patologica e Diagnostica Molecolare Careggi Hospital. A section of this specimen, stained with hematoxylin and eosin (Fig. 1), was observed by a pathologist to evaluate the percentage of cancer cells prior to performing a manual dissection of the tumor area. DNA extraction was performed using a QIAamp® DNA Mini kit (Qiagen, Hilden, Germany), and exon 2 (codons 12 and 13) of the KRAS gene was amplified by polymerase chain reaction (PCR) using the following designed primers: forward, 5'-GGTGGAGTATTTGATAGTGTAT-3' and reverse, 5'-AGAATGGTCCTGCACCAGTAA-3'. PCR was performed in a final volume of 25 µl under the following conditions: 1X buffer, 3 mmol/l magnesium chloride, 200 µmol/l deoxyribonucleotide phosphates (all from Applied Biosystems, manufactured by Roche, Branchburg, NJ, USA), 12.5 pmol of each primer (Sigma Aldrich, St. Louis, MO, USA), 200 ng/µl DNA and 1.5 U Taq DNA polymerase (Applied Biosystems manufactured by Roche). After being subjected to an initial melting temperature of 94°C for 2 min, the reaction mixture underwent 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 65°C for 30 sec, followed by a final 72°C extension step for 2 min. Prior to sequencing, the PCR products were stained with ethidium bromide and visualized on a UV transilluminator following 2% gel electrophoresis. The PCR products were then sequenced using the BigDye Terminator v.1.1 sequencing kit and an ABI Prism 310 genetic analyzer (both from Applied BioSystems, Foster City, CA, USA) (8).

Results

The percentage of sample tumor cells was evaluated by the pathologist and estimated to be ~60%. The DNA quality was evaluated by spectrophotometer analysis and the A260/A280 ratio was 1.8. The coexistence of two mutations, p.G12D and p.G12V, in the same codon (codon 12) of the KRAS gene was observed by molecular biologists in two independent PCR products and demonstrated by sense and anti-sense sequence analysis of the fragments. The DNA amplified sequence of the KRAS gene was compared with the wild-type KRAS sequence.

Discussion

Colorectal cancer is the third most commonly diagnosed type of cancer and the third leading cause of cancer mortality in men and women. With the development of drugs, including irinotecan and oxaliplatin, and targeted therapies, including cetuximab and bevacizumab therapy, the median survival has increased to >20 months. Several studies have shown that KRAS mutations in primary tumors predict resistance to anti-EGFR antibodies (9-11), and thus only patients with wild-type KRAS tumors (~60% of patients) are eligible for anti-EGFR therapy.

Although the results of the KRAS mutational analysis of the primary tumor usually match the metastases, in a minority of cases (5-10%), the KRAS mutational status is heterogeneous between the primary tumor and metastases (2,12-15). These observations may reflect the increased genetic instability in cells that progressively acquire mutations or the presence of a heterogeneous group of neoplastic cells inside the tumor (16,17). In addition, few studies have observed the coexistence of more than one mutation in the KRAS gene within the same colorectal tumor, correlating this type of alteration with clinical and morphological features (4-7).

The present study reports a case of the coexistence of two mutations - p.G12S (GGT>AGT) and p.G13D (GGC>GAC), in exon 2 of the KRAS gene - in the same selected tumor area, thus demonstrating the existence of intratumoral heterogeneity. Data in the literature suggest that multiple mutations in the KRAS gene are infrequent, representing 2.1% of mutations in colorectal cancer (18). The majority of co-mutations in the KRAS gene affect only one codon

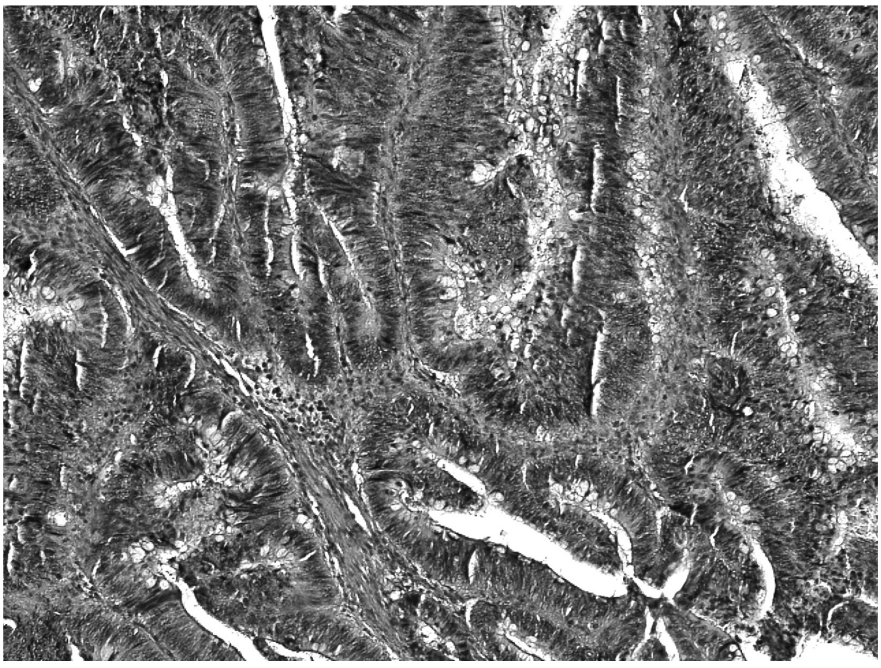

Figure 1. Section of the specimen, stained with hematoxylin and eosin.

(59%), mainly codon 12, although co-mutations may affect codons 12 and 13 simultaneously (18). The most frequently altered amino acid sequences involved in these co-mutations are GAT (in codon 12) and GAC (in codon 13) (18).

Due to the scarcity of data in the literature, the clinical implications and prognostic significance of multiple KRAS mutations remain unknown, although associations with advanced clinical stage and aggressive clinical course have been reported (4-7, 19). The present case was characterized by an aggressive clinical course with the development of early liver metastases despite the administration of neoadjuvant chemo-radiotherapy. Whether this aggressiveness was due to the coexistence of multiple mutations or a specific single mutation is a matter for debate.

It has been shown that not all KRAS mutations have the same prognostic relevance. A meta-analysis demonstrated that a p.G12S mutation at codon 12 in the KRAS gene increases the risk of recurrence or mortality in patients with colorectal cancer (17, 20-21), unlike other KRAS mutations that have only a moderate, non-significant effect on overall survival (22). These data are consistent with experimental evidence showing that valine mutations produce proteins with different behavior than other mutated KRAS proteins (12). The lower affinity of GTP to p.G12D allows p.G12D to escape from the oncogenic GTP-bound state, whereas GTP that is tightly bound to p.G12V generates a more persistent, potentially oncogenic signal. Furthermore, differences in the effector region of p.G12D and p.G12V may modify interactions with downstream signaling molecules (12).

In the present case the coexistence of these two mutations, p.G12S and p.G13D, may have had an almost (omettere?) different clinical relevance to patient prognosis (20,22). The effect of various KRAS mutations on overall survival may be explained by the fact that the heterogeneity of the various KRAS mutations in colorectal cancer may differ in carcinogenic potential. This may account for the selection of a new clone with a p.G12S mutation and more aggressive behavior, alongside the pre-existing p.G13D clone.

Hence, the great challenge is to detect all mutations present in tumors. It has been suggested that a DNA tumoral mix obtained from different tumor areas may increase the detection rate of mutations, including multiple mutations (2, 23). This is consistent with the current theory that tumors show significant intratumoral heterogeneity, characterized by separate heterogeneous somatic mutations and chromosomal aberrations (1). Such genetic heterogeneity may also cause heterogeneity in terms of radiosensitivity (24), which will greatly affect the choice of the most appropriate treatment option when the disease is treatable with radiotherapy alone or combined with chemotherapy or biological drugs (25, 26).

In conclusion, the present study underlines the importance of intratumoral heterogeneity, as supported by the current data (1, 2) in which tumors may be polyclonal with a mixture of cell populations harboring varying mutations. The coexistence of distinct clones within a tumor may have profound clinical implications for disease progression and therapeutic response. In particular, the present case appears to support the hypothesis that the presence of multiple mutations in codon 12 is associated with a more aggressive disease not responding to chemotherapy.

References

1. Gerlinger M, Rowan AJ, Horswell S, *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med 2012; 366: 883-92.
2. Baldus SE, Schaefer KL, Engers R, *et al.* Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clin Cancer Res 2010; 16: 790-9.
3. Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta 2010; 1805: 105-17.
4. 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: 508-15.
5. Jönsson M, Ekstrand A, Edekling T, *et al.* Experiences from treatment-predictive KRAS testing; high mutation frequency in rectal cancers from females and concurrent mutations in the same tumor. BMC Clin Pathol 2009; 9: 8.
6. Moerkerk P, Arends JW, van Driel M, *et al.* Type and number of Ki-ras point mutations relate to stage of human colorectal cancer. Cancer Res 1994; 54: 3376-8.
7. Sameer AS, ul Rehman S, Pandith AA, *et al.* Molecular gate keepers succumb to gene aberrations in colorectal cancer in Kashmiri population, revealing a high incidence area. Saudi J Gastroenterol 2009; 15: 244-52.

8. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463-7.
9. 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: 3992-5.
10. 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: 1626-34.
11. Karapetis CS, Khambata-Ford S, Jonker DJ, *et al.* K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008; 359: 1757-65.
12. Al-Mulla F, Going JJ, Sowden ET, *et al.* Heterogeneity of mutant versus wild-type Ki-ras in primary and metastatic colorectal carcinomas, and association of codon-12 valine with early mortality. J Pathol 1998; 185: 130-8.
13. Albanese I, Scibetta AG, Migliavacca M, *et al.* Heterogeneity within and between primary colorectal carcinomas and matched metastases as revealed by analysis of Ki-ras and p53 mutations. Biochem Biophys Res Commun 2004; 325: 784-91.
14. Mancuso A, Sollami R, Recine F, *et al.* Patient with colorectal cancer with heterogeneous KRAS molecular status responding to cetuximab-based chemotherapy. J Clin Oncol 2010; 28: e756-e758.
15. Lamy A, Blanchard F, Le Pessot F, *et al.* Metastatic colorectal cancer KRAS genotyping in routine practice: results and pitfalls. Mod Pathol 2011; 24: 1090-100.
16. Bouchahda M, Karaboué A, Saffroy R, *et al.* Acquired KRAS mutations during progression of colorectal cancer metastases: possible implications for therapy and prognosis. Cancer Chemother Pharmacol 2010; 66: 605-9.
17. 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: 675-84.
18. Macedo MP, Andrade Lde B, Coudry R, *et al.* Multiple mutations in the Kras gene in colorectal cancer: review of the literature with two case reports. Int J Colorectal Dis 2011; 26: 1241-8.
19. Perou CM, Sørlie T, Eisen MB, *et al.* Molecular portraits of human breast tumours. Nature. 2000; 406: 747-52.
20. Sørlie T, Perou CM, Tibshirani R, *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 2001; 98: 10869-74.
21. Fialkow PJ. Clonal origin of human tumors. Annu Rev Med 1979; 30: 135-43.
22. Winder T, Mündlein A, Rhomberg S, *et al.* Different types of K-Ras mutations are conversely associated with overall survival in patients with colorectal cancer. Oncol Rep 2009; 21: 1283-7.
23. Richman SD, Chambers P, Seymour MT, *et al.* Intra-tumoral heterogeneity of KRAS and BRAF mutation status in patients with advanced colorectal cancer (aCRC) and cost-effectiveness of multiple sample testing. Anal Cell Pathol (Amst) 2011; 34: 61-6.
24. Spitzner M, Emons G, Kramer F, *et al.* A gene expression signature for chemoradiosensitivity of colorectal cancer cells. Int J Radiat Oncol Biol Phys 2010; 78: 1184-92.
25. Pedicini P, Nappi A, Strigari L, *et al.* Correlation between EGFr expression and accelerated proliferation during radiotherapy of head and neck squamous cell carcinoma. Radiat Oncol 2012; 7: 143.
26. Pedicini P, Caivano R, Jereczek-Fossa BA, *et al.* Modelling the correlation between EGFr expression and tumor cell radiosensitivity, and combined treatments of radiation and monoclonal antibody EGFr inhibitors. Theor Biol Med Model 2012; 9: 23.

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Address: Antonio Taddei
Secon Unit of General and Emergency Surgery
Careggi Teaching Hospital,
Florence, Italy
E-mail: antonio.taddei@unifi.it