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Case study

KRAS and 2 rare *PI3KCA* mutations coexisting in a metastatic colorectal cancer patient with aggressive and resistant disease

Alessandra Tessitore PhD[a,*], Gemma Bruera MD, PhD[a,b], Valentina Mastroiaco MD[a], Katia Cannita MD, PhD[c], Alessio Cortellini MD[a,c], Valentina Cocciolone MD[a], Antonella Dal Mas PhD[d], Giuseppe Calvisi MD[d], Francesca Zazzeroni PhD[a], Corrado Ficorella MD[a,c], Enrico Ricevuto MD[a,b], Edoardo Alesse MD, PhD[a]

[a]*Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, 67100 L'Aquila, Italy*
[b]*Oncology Territorial Care, S. Salvatore Hospital, Oncology Network ASL1 Abruzzo, University of L'Aquila, 67100 L'Aquila, Italy*
[c]*Medical Oncology Unit, S. Salvatore Hospital, 67100, L'Aquila, Italy*
[d]*Pathology Unit, S. Salvatore Hospital, 67100, L'Aquila, Italy*

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Summary We describe a metastatic colorectal cancer patient, treated with first-line 5-fluorouracil, irinotecan, bevacizumab, and oxaliplatin (FIr-BFOx) therapy, with aggressive and resistant disease. *KRAS*, *NRAS*, *BRAF*, and *PI3KCA* were analyzed in primary tumor and liver metastasis. *KRAS* c.34G>A mutation was detected in primary tumor and liver metastasis, which additionally revealed 2 rare *PI3KCA* mutations (c.1633G>C and c.1645G>C). The c.1645G>C was never reported in colorectal cancer. Akt/p-Akt^{Ser473}, phosphatase and tensin homolog, mismatch repair, and epidermal growth factor receptor expression was evaluated. Normal mismatch repair and epidermal growth factor receptor expression was detected. Akt was shown by primary tumor and liver metastasis, whereas p-Akt^{Ser473} was identified only in the latter, despite positive phosphatase and tensin homolog expression. Patient showed 7 months of progression-free survival and 15 months of overall survival, lower than median values reported in *KRAS* exon 2–mutant patients treated with the same therapy. Results lead to the hypothesis of a putative role of these mutations in worsening of the disease and are open to further confirmatory studies.

* Corresponding author at: Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, Via Vetoio, Coppito 2, 67100 L'Aquila, Italy.
E-mail addresses: alessandra.tessitore@univaq.it (A. Tessitore), gemma.gbb@gmail.com (G. Bruera), valentina.mastroiaco@graduate.univaq.it (V. Mastroiaco), kcannita@gmail.com (K. Cannita), alessiocortellini@gmail.com (A. Cortellini), antonella.dalmas@gmail.com (A. Dal Mas), GCalvisi@asl1abruzzo.it (G. Calvisi), francesca.zazzeroni@univaq.it (F. Zazzeroni), corrado.ficorella@univaq.it (C. Ficorella), enrico.ricevuto@univaq.it (E. Ricevuto), edoardo.alesse@univaq.it (E. Alesse).

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide. Mutations in several genes are described in CRC: *KRAS* (30%-40% of cases), *TP53* (30%), *PI3KCA* (10%-30%), *BRAF* (8%-15%), *PTEN* (5%-15%), and *NRAS* (1%-6%) [1]. In the last decade, the identification of predictive/prognostic biomarkers has

improved the management of the disease [2]. Vascular endothelial growth factor (VEGF)– and epidermal growth factor receptor (EGFR)–activated signaling has been largely analyzed: 3 monoclonal antibodies, targeting EGFR (cetuximab and panitumumab) or soluble VEGF-A (bevacizumab), are in use for metastatic CRC (mCRC) [3]. *KRAS* mutations occur in early carcinogenesis and affect some hotspot codons, mainly in exon 2 (codons 12 and 13) and, less frequently, in exons 3-4 [1].

In the last few years, it has been established that the presence of *KRAS* or *NRAS* activating mutations in exons 2-4 circumvents the use of anti-EGFR [4]. On the other hand, it is known that a percentage of wild-type *KRAS*/*NRAS* patients would not benefit from EGFR inhibitors [5]. Therefore, several studies are evaluating the presence of mutations in other genes coding for proteins downstream from EGFR/RAS to define their putative impact on cancer development, prognosis, and therapy. Two among them are *BRAF* and *PI3KCA*, which could, when mutant, affect the response to EGFR inhibitors [6]. *BRAF* encodes a serine threonine kinase of the RAF family, whose pathogenic mutations are especially in exons 11 and 15. The presence of V600E substitution may limit the efficacy of anti-EGFR therapy, although some large randomized trials showed that *BRAF* mutations cannot be considered as predictive biomarkers [7,8]. On the other hand, it is known that anti-VEGF, designed to impair VEGF function, can be administered independently of *KRAS* and *BRAF* status [3].

PI3KCA encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K) involved in cell growth, proliferation, survival, and apoptosis. PI3K acts downstream EGFR by phosphorylating phosphatidylinositol 4-5 biphosphate to phosphatidylinositol 3-4-5 triphosphate and by activating downstream signaling events (protein kinase B [Akt] phosphorylation and mammalian target of rapamycin). Conversely, phosphatase and tensin homolog (PTEN) counteracts this mechanism and maintains the homeostasis of PI3K-AKT signaling in response to EGFR or other tyrosine kinase receptors by dephosphorylating phosphatidylinositol 3-4-5 triphosphate. When mutant, the *PI3KCA* catalytic subunit is constitutively active, with consequent hyperactivation of the PI3K-AKT pathway. The same effect occurs due to PTEN loss [9]. Mutations in exons 9 and 20 are responsible for most of the *PI3KCA* gene lesions in CRC. The prognostic/predictive role of *PI3KCA* gene mutations is not clearly defined yet, and the potential impact of even different molecular subgroups, characterized by the presence of mutations in *PI3KCA*, *RAS*, and *BRAF* genes, has been analyzed [10]. In this report, we describe *KRAS* and 2 rare *PI3KCA* mutations in an early onset mCRC patient who was treated with one of the most effective therapeutic regimens (5-fluorouracil, irinotecan, bevacizumab, oxaliplatin [FIr-B/FOx]) and showed very aggressive and resistant disease.

Table Activity and efficacy data of FIr-B/FOx schedule according to *KRAS* exon 2 genotype ([12] updated version)

	Overall	*KRAS* wild-type	*KRAS* mutant
No. of patients	69 (100)	32 (46)	37 (54)
ORR, % C.I.	77 ± 10	91 ± 10	64 ± 17
PFS, mo (range)	13 (1+-94+)	14 (4-94+)	11 (1+-60+)
OS, mo (range)	31 (1+-94+)	38 (8-94+)	20 (1+-60+)

Abbreviation: ORR, objective response rate.

2. Case report

2.1. Patient's clinical history

The case of a 55-year-old mCRC Italian man with synchronous liver, lung, and lymph node metastases and positive familial history is described. Patient underwent right hemicolectomy and partial resection of liver metastases. Afterward, he was treated at the Medical Oncology Unit of S. Salvatore Hospital, L'Aquila. The histological examination of the right hemicolectomy and resection of liver metastases showed an ulcerative mass of 5.3 cm at the ileocecal valve penetrating the wall to create a nodular lesion until the serosa. The histological feature was moderately differentiated (G2) mucoid adenocarcinoma (components: mucoid 60%, tubulopapillary 40%) with large areas of necrosis infiltrating the colon wall up to pericolic fat, with neoplastic permeation of some lymphatic vessels and veins, and sparse peritumoral lymphocytic reaction. Metastases were detected in 9 of 29 regional lymph nodes (1 node, 4-cm maximum dimension). Margins, rings, and omentum were negative for neoplastic cells. Three hepatic nodules of 0.7, 1, and 1.5 cm were resected and classified as liver metastases from mucoid adenocarcinoma. Pathological stage was pT3 pN2 pM1.

Because of the presence of *KRAS* c.34G>A mutation, hereafter described, the patient was treated with first-line intensive chemotherapy plus bevacizumab, according to FIr-B/FOx schedule, for 6 cycles in the expanded clinical program [11]. Clinical evaluation of response was planned by computed tomographic scan. Positron emission tomography was added based on investigators' assessment. Patient showed aggressive and resistant disease, with radiologically stable disease as the best response, and 7 months of progression-free survival (PFS). Patient was subjected to second-line chemotherapy plus bevacizumab beyond progression and to transarterial chemoembolization of hepatic lesions without benefit. Overall survival (OS) was 15 months (death), lower than those reported for overall *KRAS* exon 2–mutant patients treated with the same regimen in the same center (Table; PFS 11 months and OS 20 months) [12].

2.2. *KRAS*, *NRAS*, *BRAF*, and *PI3KCA* gene analysis

Fresh or formalin-fixed, paraffin-embedded (FFPE) tumor specimens collected at diagnosis were analyzed. Contiguous sections 3 μm thick from the interior of the primary tumor

FFPE tissue block were cut. Hematoxylin and eosin staining was performed to assess the presence and amount of tumor cells. Gross liver metastasis, histologically confirmed, was immediately frozen at −80°C. Genomic DNA was extracted (QiAmp or QiAmp FFPE DNA extraction kit, Qiagen, Hilden, Germany) and quantified by Nanodrop (Thermo Fisher, Waltham, MA). Exons 2, 3, and 4 of *KRAS* and *NRAS* genes; exon 15 of *BRAF*; and exons 9 and 20 of *PI3KCA* were amplified by using 100 ng of genomic DNA (primers in Supplementary Table S1). Amplified products were purified and sequenced (Big Dye v3.1, Thermo Fisher, Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Sequencing reactions were performed twice, using DNAs from two different extractions. No *NRAS* and *BRAF* mutations were detected. *KRAS* exon 2 c.34 G>A (G12S) mutation was detected in liver metastasis and primary tumor (Fig. 1A and B). Two *PI3KCA* exon 9 missense substitutions, c.1633 G>C (E545Q) and c.1645 G>C (D549H), were identified in liver metastasis and not in primary tumor (Fig. 1D and C). To confirm *PI3KCA* mutations, monoallelic sequencing was performed (pGEM-T easy vector, Promega, Madison, WI). Both mutations were in the same allele (Fig. 1E) in a well-conserved region among metazoans (Fig. 1F).

2.3. Immunohistochemistry

Serial 3-μm sections from FFPE tissues were deparaffinized in bio-clear (Bio-Optica, Milano, Italy) for 20 minutes and hydrated by passing through a graded series of ethanol and water. Antigen retrieval was carried out in a microwave (95°C, 15 minutes) by using unmasking citrate buffer. After peroxidase blocking, sections were incubated with 1:100 anti-Akt (Cell Signaling Technology, Danvers, MA, 9272) and 1:50 anti–phospho-Akt (Ser473) (Cell Signaling, 3787) for 20 minutes. Then, incubation with secondary antibody (EnvisionFlex HRP, Dako, Agilent Technologies Italia, Cernusco sul Naviglio, Milano) was performed (20 minutes), and DAB plus chromogen substrate (Dako) was added. After counterstaining with Carazzi hematoxylin (Dako), the sections were dehydrated and mounted. PTEN analysis was performed by using primary anti-PTEN (Dako clone 6H2.1, for diagnostic use) based on manufacturer's instructions. For mismatch repair, primary MLH1 (Dako clone ES05), MSH2 (Dako clone FE11), and MSH6 (Dako clone EP49) antibodies were used. EGFR expression was evaluated by using anti-EGFR (pharmDx kit Dako) according to the manufacturer's instructions. As a control, primary antibodies were omitted, and

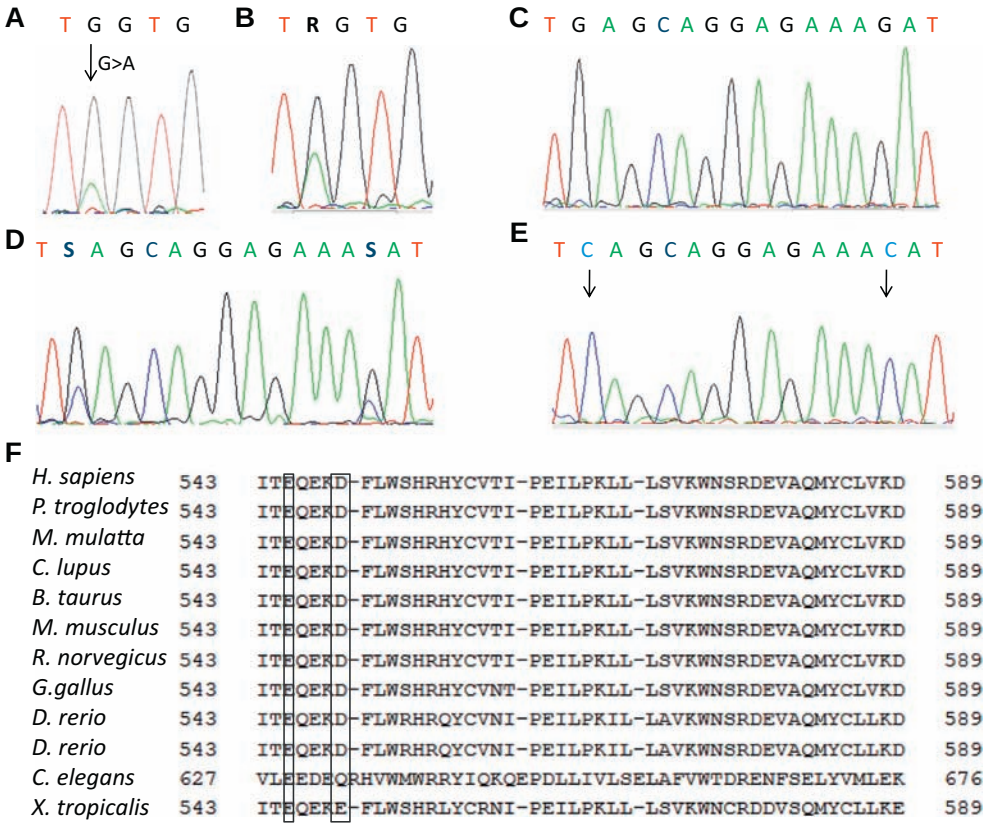

Fig. 1 Sequencing analysis of primary tumor and liver metastasis. Electropherograms of *KRAS* exon 2 and *PI3KCA* exon 9 showing *KRAS* c.34G>A (G12S) mutation in primary tumor (A) and liver metastasis (B), wild-type *PI3KCA* in primary tumor (C), and *PI3KCA* c.1633G>C (E545Q) and c.1645G>C (D549H) nucleotide substitutions in liver metastasis (D). E, Liver metastasis monoallelic sequencing with double-mutant *PI3KCA*. *PI3KCA* sequence alignments among species (results from Homologene). F, Amino acids affect by nucleotide substitutions marked with box.

sections from sample and controls (breast and CRC) were incubated only with secondary antibodies. Images were collected with a Nikon E200 (Nikon Corp., Tokyo, Japan) microscope provided by DS-L1-5M device. Primary tumor showed MLH1, MSH2, MSH6, and EGFR normal expression in both tumor components (data not shown). Primary tumor and, more markedly, liver metastasis showed Akt expression. Foci of cells with p-Akt^{S473} immunostaining were exclusively detected in hepatic metastasis and not in primary tumor (Fig. 2). PTEN expression was revealed in liver metastasis (approximately 90% of cells) with score 2 (semiquantitative scoring as reported by Sakr R. A., *Appl Immunohistochem Mol Morphol*, 2010 [13]) (Fig. 2).

3. Discussion

We report c.34G>A missense mutation, with predicted G12S change, in primary tumor and liver metastasis from an early onset mCRC. This mutation, in the P-loop region of G domain, was already classified as somatic in CRC, with 5% frequency among KRAS-mutant CRCs (Sanger COSMIC [Catalogue of Somatic Mutations in Cancer] database). *KRAS* mutations entail reduced sensitivity to anti-EGFR, but little is known about the role of specific lesions. In this regard, in a retrospective analysis of early CRCs, *KRAS* exon 2 mutations, especially c.35G>A (G12D), seem related to worse disease-free survival and OS, increasing the risk of recurrence and death particularly in Dukes C patients [14].

Beyond *KRAS* c.34G>A, very peculiar *PI3KCA* substitutions in exon 9 (c.1633G>C E545Q and c.1645G>C D549H) were exclusively detected in liver metastasis. Both transitions were in the highly conserved functional helical domain, at the level of hotspot codons whose amino acid products are well conserved among metazoans. The c.1633G>C somatic mutation is reported with very low frequency in CRCs (0.3%, COSMIC), breast cancers (<1%), and lung cancers (0.5%) (mycancergenome.org). No c.1633G>C entries are

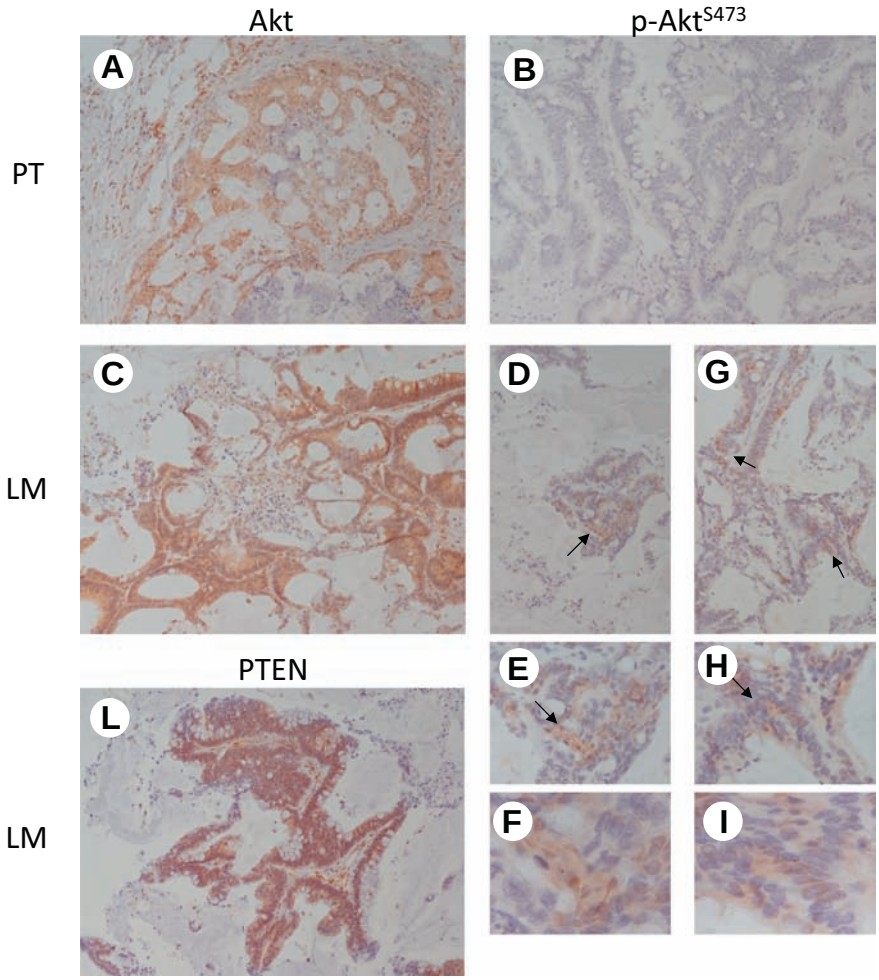

Fig. 2 Akt, p-Akt^{S437}, and PTEN expression. Positive Akt expression in primary tumor (PT) (A) and, more marked, liver metastasis (LM) (C). P-Akt was not expressed by primary tumor (B), whereas foci of positive cells were detected in liver metastasis (D-I). Positive PTEN expression in liver metastasis (L). A, B, C, D, G, and L: original magnification ×10; E and H (D and G details): ×40; F and I (H and F details, arrow): ×100.

reported in the HGMD Professional and NHLBI Exome Sequencing Project EVS databases. The c.1645G>C was never described in CRC, but the same position was affected by c.1645G>A mutation (D549N), with 0.4% frequency in PI3KCA-mutant CRCs. COSMIC database and studies reported c.1645 G > C variant in 1 of 140 cases of cervix squamous cell carcinoma, 1 of 90 hepatocarcinomas, and 1 of 149 salivary gland carcinomas [15-17].

No clear information about the function of the PI3KCA variants here identified is known, but the PolyPhen-2 tool predicts them as "probably damaging" with 0.959 (545Q) and 0.984 (549H) scores. Provean and SIFT tools classify them as "deleterious" and "damaging" as well. To preliminarily assess possible PI3K-AKT pathway activation, immunohistochemical analysis was performed. Akt expression was revealed in both primary tumor and metastasis; conversely, several foci of activated p-Akt–expressing cells were detected exclusively in liver metastasis and not in primary tumor lacking *PI3KCA* mutations. Hepatic metastasis showed PTEN expression, whose physiological role is to counteract the PI3K-AKT signaling pathway. This aspect would imply that the conjoint *PI3KCA* mutations might play a role in enhancing PI3K-AKT pathway activation. The presence of phosphorylated Akt, detected only in liver metastasis cell foci, may lead to the hypothesis that PTEN dephosphorylating activity is not completely effective. According to the previous considerations, the patient had worse prognosis, with 7 months of PFS and 15 months of OS (death), slightly inferior compared to median PFS-OS reported in *KRAS* exon 2–mutant patients treated with the same first-line intensive FIr-B/FOx regimen (11 and 20 months, respectively).

In conclusion, this report describes a peculiar early onset mCRC case showing *KRAS* and 2 very rare *PI3KCA* mutations in liver metastasis. The patient was resistant to one of the most effective therapies. This leads to the hypothesis that the association of *KRAS* and the very infrequent *PI3KCA* mutations might play a role in enhancing the aggressiveness of the disease and, possibly, in reducing the sensitivity to the therapy. Functional tests are necessary to shed light about the real significance of the above-described mutations, but this case opens to more in-depth studies focused on specifically characterizing molecular factors to be potentially evaluated in CRC management.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humpath.2018.01.021.

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