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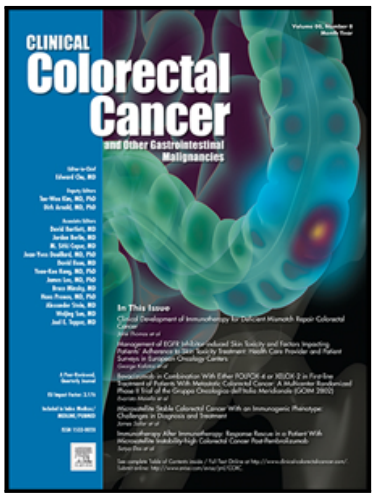

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L.P Guerrera , S Napolitano , V De Falco , E.F Giunta , P.P Vitiello , A.G. Gravina , G Suarato , A Perrone , R Napolitano , E Martinelli , F Ciardiello , T Troiani

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Multiple acquired mutations captured by liquid biopsy in the EGFR addicted metastatic colorectal cancer.

Guerrera L.P[1], Napolitano S[1], De Falco V[1], Giunta E.F[1], Vitiello P.P[2], Gravina A.G.[3], Suarato G[1], Perrone A[1], Napolitano R[1], Martinelli E[1], Ciardiello F[1], Troiani T[1]

[1]Oncologia Medica, Dipartimento di Medicina di Precisione, Università degli Studi della Campania Luigi Vanvitelli, Via S. Pansini 5, 80131 Napoli, Italia.
[2]Dipartimento di Oncologia, Università di Torino, Strada prov. 142 Km 3.95, 10060, Candiolo, Italia
[3]Unità di Epatogastroenterologia, Dipartimento di Medicina di Precisione. Università degli Studi della Campania Luigi Vanvitelli, Via S. Pansini 5, 80131 Napoli, Italia.

[*]Corresponding author: Teresa Troiani, MD, PhD, Dipartimento di Medicina di Precisione, Università degli Studi della Campania Luigi Vanvitelli, Via S. Pansini 5, 80131 Naples, Italy.
Tel: +390815666628; Fax: +390815666732; e-mail: troiani.teresa@yahoo.it; teresa.troiani@unicampania.it

Clinical practice points

- Metastatic colorectal cancer is one of the most common causes of cancer death worldwide.
- Primary and acquired resistance mechanisms to anti EGFR treatment is a challenging topic with several clinical implications
- Primary resistance is defined by the presence of activating mutations in BRAF and RAS genes before treatment initiation, while acquired resistance refers to the selection of pre-existing mutant clones or de novo acquisition of mutations under the pressure of anti EGFR treatment.
- Testing mutations in RAS and BRAF genes as predictive biomarkers is mandatory.
- Liquid biopsy has acquired growing importance and showed to be reliable when compared to tissue NGS.
- Liquid biopsy offers a full overview of the genetic landscape of the disease, overcoming spatial and temporal heterogeneity, when compared to tissue biopsy.
- Liquid biopsy can be used to capture the changes in biology of cancer cells under the selective pressure of targeted agents over time.
- Using complementary techniques allows to increase the diagnostic power and the biological significance of the results.

Keywords

metastatic colorectal cancer, primary and acquired resistance, EGFR mutations, liquid biopsy, comprehensive genome profiling

Introduction

Metastatic colorectal cancer (mCRC) is one of the most common causes of cancer death worldwide. (1) Monoclonal antibodies directed against EGFR dramatically changed its management and significantly prolonged overall outcome of mCRC patients compared to chemotherapy alone (2).

Mutations in *RAS* and *BRAF* genes have extensively been described as the main causes of both primary and acquired resistance to anti-EGFR agents, and their testing as predictive biomarkers is mandatory (2-5), traditionally assessed by polymerase chain reaction (PCR) or next generation sequencing (NGS). Primary resistance is defined by the presence of activating mutations in these genes before treatment initiation, while acquired resistance refers to the selection of pre-existing mutant clones or de novo acquisition of mutations under the pressure of anti EGFR treatment (6). Comprehensive genomic profiling (CGP) is restricted to research purposes in high-volume centers to assess further target alterations such as *HER2* amplification and *NTRK1/2* rearrangements.

In the last years, liquid biopsy has acquired a growing importance in mCRC. Its main advantages over tissue biopsy are the ability to obtain a full overview of the genetic landscape of the disease, overcoming spatial and temporal heterogeneity as well as its easy repeatability over time due to non-invasiveness, compared to standard tissue biopsy. (7) Moreover, the advent of fully automated platforms allows for a significant rapidity for the clinical decision-making in oncology. (8) As previously shown by our group, testing *RAS* and *BRAF* mutations using the automated quantitative polymerase chain reaction (qPCR)-based Idylla^{TM} Biocartis is feasible and reliable in mCRC patients when compared with tissue-based NGS analyses. (9)

Case presentation

We report the case of a seventy-four-year-old female who presented to our department for a metastatic colorectal cancer, after a radical left hemicolectomy performed in July 2014.

In February 2019, for abdominal pain and a slight variation in her bowel movements, she underwent a CT scan that showed multiple liver and pulmonary metastases, abdominal para-aortic nodal enlargement and peritoneal carcinosis. A transcutaneous liver biopsy confirmed the diagnosis of a colorectal adenocarcinoma metastasis.

The patient was enrolled in our ongoing molecular study using the CGP FoundationOne (F1) (10): baseline NGS analysis showed the tumour was wild type for the canonical mutations in *RAS* and *BRAF* genes, *HER2* negative and microsatellite stable (MSS). Tumour mutational burden (TMB) was assessed at 6 mutations/ Mb.

Being liquid biopsy an effective experimental tool available in our institution, we adopted it periodically through two different techniques: Idylla™ Biocartis (qPCR-based) and FoundationOne Liquid (NGS-based).

The qPCR-based ctDNA analysis (Idylla™ Biocartis) was performed in March 2019 and confirmed the wild type status in *KRAS, NRAS* and *BRAF* genes. In March 2019, the patient started a first line standard therapy with FOLFOX plus panitumumab, reporting a partial response at the first CT scan evaluation after six cycles (June 2019) and further reduction of the liver target lesion after additional 6 cycles (September 2019). After that, maintenance therapy with 5-fluorouracil plus Panitumumab was started.

In January 2020, after 7 cycles of maintenance treatment, CT scan revealed a significant liver progression with several measurable lesions detectable. CEA and CA 19.9 levels also increased dramatically.

The qPCR-based ctDNA evaluation in January 2020 detected several mutations: *KRAS* G12A, G12C, G12R and G12V alterations, as well as *NRAS* Q61L and Q61H mutations and *BRAF* V600E mutation. (Figure 1A/B; see Table 1 for related circulating mutational fraction, CMF, values) As disease progressed dramatically and the molecular status changed, the patient started a conventional second line therapy with FOLFIRI plus bevacizumab.

In February 2020, NGS analysis on cfDNA (F1) was performed. The same mutations in *KRAS* gene (G12A, G12C, G12R and G12V) were detected, together with *NRAS* Q61L. (Figure 1C, see Table 2 for related mutation allele fraction, MAF, values) No mutation in *BRAF* gene was described. *TP53* and *APC* splice site mutations were confirmed.

Interestingly, several *EGFR* alterations previously not reported on the baseline tissue NGS analysis were found: G465R, S464L, G465E, V441D, V441G, K489E, I491R, S464K, S464F. (Figure1D, Table 2 for related MAF values)

In March 2020, qPCR-based ctDNA evaluation was performed again: mutations in *KRAS* G12A, G12C and G12V were detected showing lower CMF values than those reported in January 2020. (Figure 1A, Table1). *KRAS* G12R alteration was not described anymore. No mutations in *NRAS* and *BRAF* were found. (Figure 1B)

In May 2020, after 6 cycles of second line therapy, CT scan showed a further volumetric increase in liver metastases, paralleled by CEA and Ca19-9 increment. The patient complained uncontrolled abdominal pain and was addressed to best supportive care, but gave her informed consent to additional liquid biopsy analyses.

At the qPCR-based ctDNA analysis, an additional *KRAS* mutation (G12D) was reported, together with G12A, G12C, G12R, and G12V. (Figure 1A, Table 1). NRAS Q61L and BRAF V600E/D mutations were described again. (Figure 1B)

At NGS analysis on cfDNA, the same *KRAS* alterations were detected except for G12R and additional ones were reported: G13D, Q61H (183A>T, 183A>C), G12L and G12F alterations. (Figure 1C, Table 2). Furthermore, *NRAS* Q61L mutation was described with a decreased MAF when compared to that of March 2020. (Figure 1C. Table 2) *EGFR* alterations previously reported were confirmed, though with a different allele frequency (decreased for G465R, I491R, S464L, G465E and K489E, increased for V441D and V441G). *EGFR* I491K substitution was a new finding. (Figure 1D, Table 2)

In July 2020 the patients deceased due to hepatic failure.

Material and methods

Two different techniques for liquid biopsy analysis were periodically used and compared: Idylla™ Biocartis and FoundationOne Liquid .

Idylla™ Biocartis is a fully automated real time-PCR based platform that evaluates the most frequent mutations of KRAS, NRAS and BRAF genes in a minimal amount of time (almost two-hours). Results are generated as Cq values for each mutation and the internal control and can be expressed using the circulating mutational fraction (CMF) that represents the relative amount of mutant DNA over total cell free DNA (cfDNA), as previously described. (9)

FoundationOne (F1) Liquid is a NGS-method for ctDNA analysis able to assess simultaneously, the four main classes of genomic alterations in 77 genes as well as microsatellite status (MS). Its results are expressed as MAF that represents the number of mutant reads divided by the total number of reads at a specific genomic position.

Discussion

In this report, we described a clinical case of a patient diagnosed with MSS mCRC who developed an impressive amount of mutations following chemotherapy plus anti-EGFR, detected using a qPCR-based platform (Idylla Biocartis) and a NGS platform (FoundationOne Liquid).

High concordance was found between the two techniques, with most mutations detected by both approaches at the corresponding time points (Table 1-2), though in the last report of May 2020, F1 Liquid identified several additional alterations (KRAS G12D, G13D, G12L, G12F and Q61H) that were not reported by qPCR analysis.

In our patient, ten different EGFR mutations were detected. (11-13) Interestingly, although the MAF values of most of them decreased during second line therapy as expected, a rise in MAF for V441G and V441D alterations (1.1% and 0.34% vs 0.54% and 0.16%, respectively) was reported as well as the emergence of I491K substitution. (Fig 1D, Table 2)

To date, not all of them have been fully characterized functionally and most of data derive from preclinical assays. EGFR 465R, 465E and S464L alterations were already related to decreased antibody binding (13-14). Less is known about the effects of EGFR S464K and S464F mutations as well as of I491K substitution (13). Finally, mutations involving V441 (V441G and V441D) have been recently identified as a prominent new cluster of EGFR ECD alterations: both seem to be responsible to interfere with the interaction between cetuximab and EGFR (15).

As already reported by Van Emburgh et al., we verified in our patient that secondary EGFR ECD alterations, either with or without RAS ones, are associated with good tumor shrinkage and longer PFS to first-line anti-EGFR-based therapies (16).

The emergence of so many acquired RAS mutations despite anti-EGFR discontinuation is a very rare finding as well as the onset of a plethora of EGFR alterations, all localized in its ectodomain. (11-12) Both these events are of particular interest as they were reported in a mCRC with a low TMB and no molecular explanation for such a high mutability, given the MSS status and the absence of mutations in polymerases genes (POLE or POLD) (17)

Recently, it was described how MSS colorectal cancer cells undergoing oncogenic deprivation are characterized by deregulation in mechanisms of DNA repair that lead to adaptive mutability, resulting in increased mutagenesis (18). This mechanism may explain the molecular pathogenesis of these many mutations after an initial response to the treatment in our case, presuming that each

mutation is related to a single independent tumoral clone and that all these mutations confer a selective advantage under the selective pressure imposed by the anti-EGFR treatment.

In conclusion, in this work we showed (a fascinating) an interesting case of how the complex biology of cancer cells under the selective pressure of targeted agents can be captured over time using liquid biopsy, that represents the landmark for the study of genomic evolution in mCRC. Moreover, we showed how the use of complementary techniques allows to increase the diagnostic power and the biological significance of the results.

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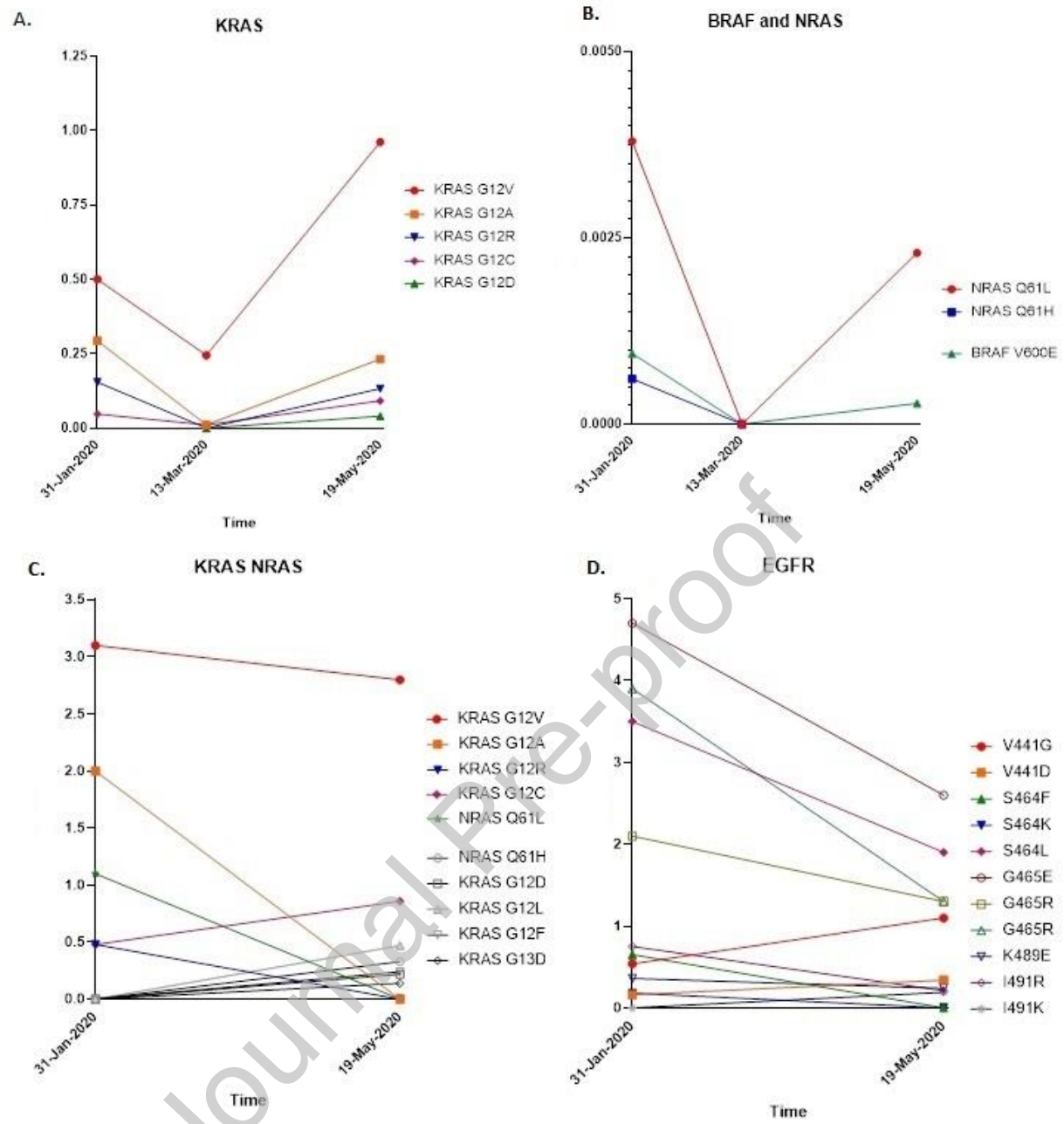

Figure 1: A, B , C, D. Herein we provide an overview of the mutations detected in the main genes analyzed through Idylla™ Biocartis and Foundation One liquid, related to CMF (Figure A/B) and MAF values (Figure C/D) respectively.

Figure 1 A/B: diagrams showing the alterations detected in the three samples analyzed through Idylla™ Biocartis are offered with results expressed as CMF. On the right (Fig. A), the image summarizes all KRAS mutations found, while on the left (Fig.B) NRAS and BRAF mutations. As already mentioned above, CMF represents the relative amount of mutant DNA over total cell free DNA (cfDNA). Most of the mutations showed a similar behaviour with a decrease in CMF values in March 2020 and a slight increase in May 2020.

Figure 1 C/D: graphs offer an overview of the main alterations detected through F1 liquid in January and May 2020, correlating them to mutation allele fraction (MAF) values. As already explained above, MAF represents the number of mutant reads divided by the total number of reads at a specific genomic position.

On the right (Fig.1C) KRAS and NRAS mutations are reported in the same graph, while on the left (Fig D) EGFR mutations are summarized. It is easy to perceive a general trend towards the reduction of MAF for most of the alterations described with a slight increase for some of them, especially KRAS ones.

Table 1: CMF values related to mutations reported by Idylla™ Biocartis. Circultating mutation fraction**(**CMF) represents the relative amount of mutant DNA over total cell free DNA (cfDNA) and is

calculated as the percentage of mutated DNA at a specific site over the internal control as follows: 2 - DCq × 100 (where DCq values express the difference between the Cq of the mutation of interest and the mean Cq of the internal control). CMF highly depends on the quantity of non-tumour circulating DNA. Being its value directly correlated with the abundance of total ctDNA as well as the mutation allele fraction (MAF), CMF might be used as a clinical surrogate of these two parameters. Herein we report CMF values related to the main mutations identified in KRAS, NRAS and BRAF genes on blood samples analysed through Idylla™ Biocartis, performed in 3 crucial phases of the natural history of disease: January 2020, when hepatic progression of disease was assessed and second line treatment was started, March 2020 and finally May 2020,when disease progressed dramatically and the patient was addressed to BSC. Interestingly, in March 2020, CMF values decreased for most of the KRAS mutations described in January (G12A, G12C, G12V) and some alterations were not detected anymore such as KRAS G12R , NRAS Q61L, NRAS Q61H and BRAF V600E. In May 2020, as disease dramatically progressed, CMF values of KRAS G12A, G12C and G12V mutations raised again, despite the withdrawal of anti-EGFR agents. Further alterations were detected such as KRAS G12 R, NRAS Q61L and BRAF V600E: interestingly, all of them had been already described in January 2020. KRAS G12D was reported for the first time. These data were concordant with clinical and imaging findings.

Data	**KRAS**	**CMF**	**NRAS**	**CMF**	**BRAF V600E**	**CMF**
31/01/2020	Test # 551	G12A: 0.294% G12C: 0.047% G12R: 0.155% G12V: 0.501%	Test # 552	Q61L: 0.0038% Q61H: 0.00061%	Test # 552	0.00095%
13/03/2020	Test # 623	G12A:0.0114% G12C: 0.0112% G12V: 0.245%	Test # 624 (neg)	X (neg)	Test # 624 (neg)	X (neg)
19/05/2020	Test # 676	G12A: 0.232% G12C:0.0918% G12R: 0.133% G12V: 0.962% G12D:0.0399%	Test # 678	Q61L: 0.0023%	Test # 678	0.00028%

Table 2: MAF values related to gene alterations described by FoundationOne Liquid. As already mentioned in Figure 1, MAF represents the number of mutant reads divided by the total number of reads at a specific genomic position. In May 2020, MAF decreased for KRAS G12V mutation, increased for G12C and remained stable for G12A. KRAS G12R alteration was not detected anymore. Interestingly, several KRAS mutations, that were not described in February, were found in May 2020 (KRAS G12D, Q61H, G13D, G12L, G12F) despite the withdrawal of anti-EGFR agent. NRAS mutation Q61L showed a slightly decreased MAF in May 2020. The most interesting finding was the detection of 10 mutations at EGFR gene in February 2020 that had not been described by NGS performed on liver biopsy collected in March 2019: six of them showed decreased MAF in May 2020, two had a slight increase in MAF values, and the last two ones were not detected anymore. Interestingly, a new alteration (EGFR I491K substitution) was reported in May 2020. Altogether these findings may be related to anti-EGFR agent pressure till January 2020 and subsequent withdrawal of the drug. However, although panitumumab was stopped, 2 alterations out of 10

showed a modest increase in MAF values and a new substitution was reported in May 2020. These latter events may be stochastic or may be related to a selective advantage determined by these alterations. The occurrence of EGFR mutations located in ECD domain may be expression of the socalled adaptive mutability, that is thought to be responsible for increased mutagenesis in tumours where it cannot be explained (low TMB, MSS, wild type POL genes). Presuming that each mutation is related to a single independent tumoral clone and that confers a selective advantage under anti-EGFR treatment pressure, this mechanism may explain the molecular pathogenesis of these many mutations after an initial response to the treatment in our case.

KRAS	**MAF-February 2020**	**MAF-May 2020**	**NRAS**	**MAF-February 2020**	**MAF-May 2020**	**EGFR**	**MAF February 2020**	**MAF May 2020**
G12V ex 2 cod 12	3,10%	2,80%	**Q61L ex3 cod61**	1,1%	0,6%	**G465R 1393 G>C**	3,90%	1,30%
G12A ex2 cod12	2,00%	2,00%				**G465R 1393 G>A**	2,10%	1,30%
G12C ex2 cod12	0,48%	0,86%				**S464L**	3,50%	1,90%
G12R ex2 cod12	0,48%	absent				**G465E**	4,70%	2,60%
G12D ex 2 cod 12	absent	0,22%				**V441D**	0,16%	0,34%
Q61H ex 3 cod 61 (183 A>T)	absent	0,11%				**V441G**	0,54%	1,10%
G13D ex 2 cod 13	absent	O,14				**K489E**	0,36%	0,24%
G12L ex2 cod 12	absent	0,47%				**I491R**	0,75%	0,21%
Q61H ex 3 cod 61 (183 A>C)	absent	0,20%				**S464K**	0,18%	absent
G12F ex2 cod 12	absent	0,24%				**S464F**	0,65%	absent
						I491k	absent	0,21%