RAS Mutational Status in Advanced Colorectal Adenocarcinoma Treated With Anti-angiogenics: Preliminary Experience With Liquid Biopsy

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Abstract. *Aim: To determinate molecular changes in the downstream epidermal growth factor receptor signaling pathway using serial liquid biopsies in patients with metastatic colorectal tumors (mCRC) under anti-angiogenic treatment. Patients and Methods: Determination of RAS mutation in primary tissue samples from colorectal tumors was performed in the 23 patients included in the study at diagnosis using quantitative-polymerase chain reaction. Sequential mutations were studied in circulating tumor (ct) DNA obtained from plasma samples. Results: Twenty-three patients with RAS-mutated primary tumors were included. In the first ctDNA determination, 17 of these patients were found to have wild-type RAS status. Remarkably, three out of these 17 wild-type cases changed to RAS-mutated in subsequent ctDNA assays. Conclusion: Serial liquid biopsies in patients with mCRC might be a useful tool for identifying changes in the RAS mutation status in patients who had undergone previous anti-angiogenic therapy. The*

understanding of these changes might help to better define the landscape of mCRC and be the path to future randomized studies.

Colorectal cancer (CRC) is an aggressive disease associated with a poor outcome in a subset of patients. Approximately 22% of CRCs are metastatic (mCRC) at initial diagnosis and about 70% will develop metastatic relapse (1). With the arrival of directed therapies against epidermal growth factor receptor (EGFR) signaling pathway for patients without mutations in *KRAS* proto-oncogene, GTPase (*KRAS*), *NRAS* proto-oncogene, GTPase (*NRAS*) and B-RAF proto-oncogene, serine/threonine kinase (*BRAF*) genes, overall survival has increased. The use of anti-angiogenic based chemotherapy has also revolutionized mCRC treatment. However, the impact of anti-angiogenics on the molecular profile of patients with *RAS*-mutated tumors is unclear. It has been hypothesized that *KRAS*-mutated tumors treated with vascular-directed therapy might change to a wild-type *KRAS* status (2). Drug-induced hypoxia has been suggested as a possible explanation for this, and has been related to overexpression of wild-type *KRAS* (3). However, to date, only a few studies have examined in depth the consequences of these changes in the evolution of the disease.

Detection of mutation status of *RAS* genes in circulating tumor DNA (ctDNA) from blood samples seems to be a simple and non-invasive alternative to testing of primary tumors (4). It can easily be done at different time points in the course of the disease, providing information on dynamic changes occurring in the genotype of mCRC cells.

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Our hypothesis is that performing serial liquid biopsies in patients with mCRC under anti-angiogenic treatment might be a useful tool for establishing whether clonal selection occurs in patients with CRC under anti-angiogenic treatment. One of our objectives was to determine whether such changes remain over time or disappear as vascular endothelial growth factor (VEGF) inhibition is withdrawn.

Patients and Methods

We present a retrospective cohort study performed at the Infanta Sofía University Hospital in Madrid, Spain, from June 2018 to June 2019. The study was approved by the Local Ethics Committee (reference number: PI-4292).

Patients included in the study had mCRC with any mutation in RAS/RAF/mitogen-activated protein kinase: 17 patients showed mutation in *KRAS* oncoprotein and six patients in *NRAS* oncoprotein. All patients had received anti-VEGF treatment within the previous 6 months or were under such therapy at the time of the study. We performed assays of ctDNA for every patient included in the study, at the time of study inclusion and subsequently every 2 months, and finally at the time of radiological progression. A computed tomographic scan was performed every 2 months to exclude tumor progression. The Response Evaluation Criteria in Solid Tumors (RECIST) were used to establish progression of the disease (5).

Paraffin-embedded primary tumor specimens containing at least 70% tumor cells were selected for each patient. A polymerase-chain reaction (PCR) was performed to amplify 139 bp of exon 2, exon 3 and 4 in *KRAS* using specific primers. PCR primer sequences/conditions are available upon request. Further mutations in *NRAS/BRAF* were tested when no mutation in *KRAS* exons was found. When no *NRAS* mutation was found, *BRAF* V600E mutation was analyzed. The efficiency and quality of the polymerase-chain reaction (PCR) amplification were confirmed by running the products on a 2% agarose gel. A negative control containing all the components of the PCR except the template was included in each PCR reaction. DNA-amplified products were purified using a QuickStep™ 2 96-Well PCR Purification Kit (Edge BioSystems, Gaithersburg, MD, USA), according to the manufacturer's instructions. Amplification products were bidirectionally sequenced via the fluorescence dye terminator method in a multi-capillary DNA. The presence of mutation was accepted when its chromatographic peak height was 25% or higher than the peak of the wild-type reference.

For liquid biopsy, two analytic kits were used: Idylla™ ct*KRAS* Mutation Assay, which globally covered 21 mutations: seven mutations in exon 2 (codon 12 and 13), nine mutations in exon 3 (codon 59 and 61) and five mutations in exon 4 (codon 117 and 146); and Idylla™ ct*NRAS/BRAF* Mutation Assay, which covered 23 mutations: eight *NRAS* mutations in exon 2 (codon 12 and 13), six *NRAS* mutations in exon 3 (codon 59 and 61), four *NRAS* mutations in exon 4 (codon 117 and 146), and five *BRAF* mutations in exon 15 (codon 600, V600E mutation). The sensitivity (weighted average) and the sensitivity range (most prevalent *RAS* mutations) of Idylla™ ct*RAS* was 0.4% (0.2-0.5%) for *KRAS*, 1.5% (0.5-1.3%) for *NRAS* and 0.4% (0.4%) for *BRAF*.

Data were analyzed by two independent statistics experts from the European University in Madrid using IBM SPSS Statistics 23 (IBM, Armonk, NY, USA).

Table I. Baseline demographic data of patients included in the study (n=23).

Characteristic	Value
Gender, n (%)	Female 8 (34.8%) Male 15 (65.2%)
Age, years	Mean±SD 67.3±9.8
Location of primary tumor, n (%)	Rectum 9 (39.1%) Colon 13 (56.5%) Colorectum 1 (4.4%)
Primary characteristics, n (%)	Lymph node metastases 11 (47.8%) Perivascular/lymphatic invasion 8 (34.8%) Microsatellite instability 1 (4.4%)
Stage at diagnosis*	II 1 (4.3%) III 4 (17.4%) IV 18 (78.3%)
CEA	Median (IQR) 2.5 (1.3-86.6) <5 µg/l 14 (60.9%) ≥5 µg/l 9 (39.1%)
LDH	Median (IQR) 230 (173-272) <250 U/l 14 (60.9%) ≥250 U/l 9 (39.1%)
CA 19.9	Median (IQR) 23 (9-107.5) <40 U/ml 13 (56.5%) >40 U/ml 9 (39.1%) Unknown 1 (4.4%)
Liver metastasis, n (%)	Synchronous 14 (60.9%) Metachronous 2 (8.7%)
Treatment received, n (%)	Aflibercept 1 (4.3%) Regorafenib 0 (0.0%) Bevacizumab 21 (91.3%) Bevacizumab+aflibercept 1 (4.3%)
Number of chemotherapy lines prior to LB, n (%)	0 12 (52.2%) 1 7 (30.4%) 2 3 (13.0%) 3 0 (0.0%) 4 1 (4.3%)
Primary tumor mutation, n (%)	<i>KRAS</i> 17 (73.9%) <i>NRAS</i> 6 (26.1%)

CA 19.9: Cancer antigen 19.9; CEA: carcinoembryonic antigen; IQR: interquartile range; LB: liquid biopsy; LDH: lactate dehydrogenase; SD: standard deviation. *American Joint Committee on Cancer 8th edition.

Results

Twenty-three patients were included. Table I describes the demographic data of the study cohort. The mean age at diagnosis was 67.3±9.8 years. Only one patient exhibited microsatellite instability, with loss of expression of *MSH2*. Prior to liquid biopsy, 21 patients had been treated with bevacizumab, one with aflibercept, and one had received both drugs. The median time from the initiation of anti-angiogenics to the performance of the first liquid biopsy was 4 (range=1-28) months. Among primary tumors, 17 and 6 carried *KRAS* and *NRAS* mutations, respectively. No patient harbored *BRAF* mutation. The first ctDNA analysis showed

that the *RAS* mutation status had changed to wild-type in 17 patients (73.9%), whereas six patients had maintained their *RAS* oncogene mutation. Subsequent ctDNA analysis identified three additional *RAS* mutations among these 17 (17.6%) patients (*KRAS* exon 2, *KRAS G13D* in two patients and *KRAS G12R* in one patient). Interestingly, the *RAS* mutation status of these three patients returned to their initial *RAS* mutation status after a median duration of treatment with anti-angiogenic drugs of 7 (range=6-9) months. Our study was not able to determine the precise moment when the *RAS* mutational status changed, as sequential biopsies were performed on a two-month basis.

Discussion

Identification of ctDNA by liquid biopsy in mCRC tumors is a simple and convenient method for assessment of *RAS* mutation. Different studies, including the PERSEIDA (6) and the AGEO RASANC study (7), have shown excellent concordance between the *RAS* mutational status found in tissue samples of CRC and those in liquid biopsies (6-8). Therefore, it is an excellent tool for assessing molecular changes in patients with mCRC. Serial liquid biopsies performed in our cohort of patients with mCRC treated with anti-angiogenics showed changes in the *RAS* oncogene genotype in ctDNA. These results support the fact that mCRC is a dynamic condition with an evolving molecular profile over the course of the disease.

Selection of resistant clones following systemic therapy has been reported in a wide variety of tumors (9-11).

These surviving cells have a higher phenotypic plasticity and ability to grow and disseminate to distant organs (12). In our study, we observed a negative selection of *RAS*-mutant clones from plasma samples of patients treated with anti-angiogenic-based chemotherapy treatment, supporting previous data (13-15). Our results suggest that anti-VEGF treatment might play a critical role in this clonal selection.

However, our study was not able to investigate whether these changes were secondary to anti-VEGF treatment, chemotherapy, both, or just related to intrinsic genotypic alterations.

Our results support other data in the literature. Bouchahda *et al.* genotyped plasma of 16 primary *RAS*-mutated tumors from previously treated patients with mCRC. Nine patients showed wild-type *RAS* in their ctDNA. They took a step forward and offered patients with wild-type *RAS* anti-EGFR treatment (13). Likewise, Raimondi *et al.* reported the disappearance of *RAS*-mutant clones from the plasma of four patients initially carrying *RAS*-mutated primary tumors, all of which had been treated with anti-VEGF (14). These patients were also treated with anti-EGFR and some patients achieved a partial response. However, so far, the efficacy of anti-EGFR therapy in cases with wild-type *RAS* determined in ctDNA

with initially mutated tumors is unknown, and anti-EGFR treatment cannot be widely recommended for these patients until prospective randomized studies are held. Our results suggest that this area of research should be more profoundly explored and randomized studies should be performed.

The fact that 17 out of 23 of our patients experienced a selection of clones with wild-type *RAS* in our study highlights the importance of performing molecular analysis prior to the administration of further lines of therapies. Moreover, considering that three of them reverted to their previous mutated status, our study supports the fact that clonal selection seems to be a dynamic feature and performing sequential biopsies at different times during the disease course might add substantial information regarding clonal selection.

The present study has some limitations. Firstly, the small number of patients included makes it difficult to extrapolate the results and establish precise conclusions. Secondly, the probability of detecting false-negative mutated tumors is plausible when using the Idylla™ kit, as reported by Vivancos *et al.* They compared Idylla™ quantitative *ctKRAS* mutation test to OncoBEAM digital PCR assay, showing a lack of concordance in tumors with a mutated allelic fraction (MAF) below 1% (16). Unfortunately, we were unable to use OncoBEAM® or other next-generation sequencing techniques to validate our results. However, Vidal *et al.* showed the presence of MAF seems to be conditioned not by the number of metastatic sites but by the site of metastasis (17). In their study, patients with liver involvement had higher *RAS* ctDNA (*RAS* MAF) compared with those without liver metastases ($p=0.001$). In contrast, MAF from patients having only peritoneal metastases or lung metastatic involvement was much lower. In our study 14 patients had synchronous liver metastasis at the time of diagnosis and inclusion in the study. Hence, the expected higher mutated allelic fraction related to these tumors ensures the sensitivity of the Idylla™ technique. Finally, concomitant therapies (anti-angiogenics are commonly administered along with other cytostatic agents) are a potential source of bias in which *RAS* mutational changes induced might also be induced by prior chemotherapies, indistinguishable from those induced by anti-angiogenics.

Nonetheless, the results of our study support the concept that anti-angiogenic therapy might induce changes in *RAS* mutational status in patients with mCRC. Liquid biopsy is a minimally invasive and easily tool with promising results for dynamic evaluation of *RAS* mutations. It can be performed at different periods of the disease and might guide the selection of targeted treatments, such anti-EGFR therapy. Assessment of changes from *RAS*-mutated to wild-type *RAS* variants in patients with metastatic CRC and evaluation of anti-EGFR therapy in this context warrants further investigation and prospective randomized studies are required.

Conflict of Interest

On behalf of all Authors, the corresponding author states that there are no conflicts of interest.

Authors' Contributions

EC, ML-G and BGS: Designed the research. ML-G, BGS, PDD-L and CG-R: wrote the article. EC, AJG, CG-R and FZT: contributed in different ways to the writing. PDD-L, AMH and IL reviewed the article. FN and IJT-V developed the statistical analysis. BGS and JM-R performed the measurements. All Authors contributed to the article and approved the submitted version.

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