True conversions from RAS mutant to RAS wild-type in circulating tumor DNA from metastatic colorectal cancer patients as assessed by methylation and mutational signature.

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Abstract

The paucity of targeted treatments available in patients with RAS mutant colorectal cancers contributes to the poor prognosis of this patient group compared to those with RAS wild-type disease. Recent liquid biopsy-driven studies have demonstrated that RAS mutant clones might disappear in plasma during the clonal evolution of the disease, opening new unforeseen perspectives for EGFR blockade in these patients. Nevertheless, the lack of detection of RAS mutations in plasma might depend on the low amount of released circulating tumor DNA (ctDNA), making it necessary a more accurate selection of patients with true RAS mutation conversions. In this liquid biopsy-based study, we assessed RAS mutational status in initially RAS-mutant patients at the time of progressive disease from any line of therapy and investigated the incidence of true conversions to plasma RAS wild-type, comparing a colon cancer specific methylation profile with a mutational signature of ctDNA. Globally, considering either mutational panel or methylation profile as reliable tests to confirm or exclude the presence of ctDNA, the percentage of "true RAS converters" was 37.5%. In our series we observed a trend toward a better PFS in patients who received anti-EGFR as second or subsequent treatment lines compared to those who did not.

Keywords

Circulating tumor DNA, RAS mutation, colorectal cancer, EGFR blockade, methylation

1. Introduction

Our increasing knowledge on the molecular complexity of colorectal cancer has allowed to take significant steps towards precision oncology, particularly in patients with metastatic disease, with a direct effect on both progression free and overall survival [1]. Targeted therapies directed against the epidermal growth factor receptor (EGFR) pathway represent the standard of care in patients with RAS wild-type tumors. Tumor tissue genotyping is a necessary premise to exclude the presence of predictive mutations of innate resistance to anti-EGFR therapy. Metastatic colorectal cancer (mCRC) patients who initially respond to anti-EGFR monoclonal antibodies often become resistant to treatment. In at least half of the cases, drug resistance is associated to the appearance of RAS mutations, which can be detected in the blood of patients through liquid biopsies months before instrumental/clinical progression of the disease [3-5]. These observations have paved the way for so-called "adaptive" therapies based on the concept of cancer clonal evolution. In fact, several lines of evidence have shown that re-treating wild-type (wt) RAS patients with an anti-EGFR already used during a first line (the so-called "rechallenge"), can be a promising strategy, even after progression to multiple lines of treatment [6-8]. Similarly, recent evidence has been provided that the clonal evolution of mutant RAS colon cancer can lead to the negative selection of mutant RAS clones, with the appearance of a "time-window" characterized by the prevalence of wt RAS clones as assessed in plasma circulating tumor DNA (ctDNA). This phenomenon, described as "ras clearance" might represent a rationale to candidate these patients to EGFR inhibition, although its frequency seems extremely variable according to studies [9-13]. In this respect, the sensitivity of the method is of crucial importance, as the lack of RAS mutations detection in a plasma sample may depend on the low analytical sensitivity of some tests currently used in liquid biopsy, as well as on a scarse ctDNA release in some patients. A low burden of disease, encephalic or pleural progressions generally characterize patients whose tumors release low quantity of ctDNA (so-called "non- shedders"), with an increased risk of possible false negative results [14]. In these cases, the absence of mutations detected in ctDNA could lead to an erroneous interpretation of "RAS wt" disease. Thus, the use of methods able to confirm rather than exclude the presence of ctDNA in a plasma sample is imperative. In this respect, two possible approaches have been recently suggested [15]. The first, which however requires a priori knowledge of the somatic mutations present in the primary tumor tissue, would be to investigate the presence of somatic mutations in the ctDNA sample through next generation sequencing (NGS). In this way the detection of at least one somatic mutation of a gene other than RAS in the plasma would be indirect proof of the presence of sufficient ctDNA amount in the sample. Alternatively, recent evidence has been provided that cancer-specific methylated biomarkers can be used as a "normalizer" of the amount of ctDNA present in a plasma sample [16-18]. Aim of the present study was to investigate the incidence of true conversions from tissue RAS mutant to plasma RAS wt, using both NGS and a colon cancer specific methylation assay in order to discriminate" true converters" from "non-shedder patients.

Abbreviations: ctDNA: circulating tumor DNA; EGFR: epidermal growth factor receptor; IGV: integrative genomics viewer; IT-PGM: ion torrent personal genome machine; LOD: limit of detection; mCRC: metastatic colorectal cancer; NGS: next generation sequencing; PCR: polymerase chain reaction; PD: progressive disease; PFS: progression free survival; RECIST: response evaluation criteria in solid tumours; wt: wild-type.

2. Patients and methods

Forty patients treated between January 2018 and August 2020 at Policlinico Umberto I with a primary diagnosis of RAS mutant mCRC were included. Inclusion criteria were: males or females, age > 18 years; evidence of RAS/BRAF mutations as assessed in both primary tumor tissue and ctDNA at diagnosis; concordance in RAS mutational status between primary tumor and ctDNA at diagnosis; at least one previous chemotherapy regimen received; documented progressive disease on imaging; measurable disease according to Response Evaluation Criteria in Solid Tumours (RECIST criteria, vers.1.1); ECOG Performance Status ≤ 2; signed informed consent.The mutational analysis of RAS/BRAF genes on tumor tissue (obtained from biopsy or surgery) or in metastatic sites had been performed, as part of the routine diagnostic process using Ion Torrent Personal Genome Machine (IT-PGM) [19-20]. The screening of mutational status of RAS/BRAF in ctDNA had been performed through real-time polymerase chain reaction (PCR) (Idylla™, Biocartis). The system allows to rapidly detect the presence of 21 mutations in codons 12, 13, 59, 61, 117 and 146 of the KRAS gene, 18 mutations in codons 12, 13, 59, 61, 117, 146 of the NRAS gene, 5 mutations in codons 600 of the BRAF gene and 2 mutations in codons 492 of the EGFR gene, from 1 mL of plasma [21]. A blood sample (10 mL) was prospectively obtained for ctDNA analysis at the time of progressive disease (PD) from every line of therapy. Blood samples obtained at PD were centrifuged at 1500 rpm for 10 min, then plasma was removed and further centrifuged at 13,000 rpm for 1 min. Plasma samples were aliquoted and immediately screened for RAS mutational analysis through Idylla™ system. Plasma samples showing wt RAS status were further processed for IT-PGM sequencing. To this purpose, ctDNA was purified from plasma for each patient (1 mL) by Maxwell 16 system (Promega) using Maxwell RSC ccfDNA plasma kit according to the manufacturer's instructions. Eluted ctDNA quantity was assessed with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) using Qubit™ dsDNA HS Assay Kit (Thermo-Fisher Scientific). ctDNA samples were analyzed using the Oncomine™ Colon cell-free DNA Assay (Thermo Fisher Scientific) containing a single primer pool to amplify hotspots and targeted regions of fourteen genes (>240 hotspots are covered): AKT1, BRAF, CTNNB1, EGFR, ERBB2, FBXW7, GNAS, KRAS, MAP2K1, NRAS, PIK3CA, SMAD4, TP53, APC. This assay enables the analysis of single nucleotide variants and short indels that are frequently mutated in colon/gastro-intestinal cancers. By using tag sequencing technology, a limit of detection (LOD) down to 0.1% can be achieved (https://www.thermofisher.com/order/catalog/product/A31182#/A31182). Briefly, 20 ng of cfDNA input or a maximum volume of 13 µL per sample were used for libraries preparation, according to the manufacturer's instructions. Templated spheres were prepared using 100 pM of each library by using the Ion One Touch 2.0 machine (Thermo Fisher Scientific). Template-positive spheres were loaded into Ion chip 318 and sequenced by IT-PGM machine (Thermo Fisher Scientific). Sequencing data were analyzed with the Ion Torrent Suite Software (Thermo Fisher Scientific, http://github.com/iontorrent/TS) and Ion Reporter Software, according to company's recommendations. Variants were verified using the integrative genomics viewer (IGV) visualization tool (http://www.broadinstitute.org/igv/). For methylation analysis, ctDNA was purified from 1 mL of plasma by Maxwell 16 system (Promega) as described above. 20 µL of ctDNA were used for bisulfite conversion using the EZ DNA methylation Gold kit (Zymo Research), following manufacturer's protocol, with final elution in 40 µl. Bisulfite converted ctDNA was assessed for the methylation status of five genes (EYA4, GRIA4, ITGA4, MAP3K14-AS1, MSC), hypermethylated

in colorectal cancer [22]. Since no specification in the European Marketing Authorisation for Cetuximab mentions that tumor RAS testing should be determined on solid tumor tissue in order to allow for anti-EGFR administration [https://www.ema.europa.eu/en/medicines/human/EPAR/erbitux], a RAS wt ctDNA sample (as confirmed through both NGS and methylation assays) at the time of disease progression was considered suitable to allow treatment with EGFR inhibitors.

3. Results

3.1. Patients

Forty patients treated at Policlinico Umberto I between January 2018 and August 2020 for RAS mutant metastatic colorectal cancer (as assessed by both primary tissue and baseline ctDNA analysis) were prospectively enrolled at the time of PD from any previous line of treatment. There were 29 males and 11 females, average age 60 (range 42-78). Twenty patients were enrolled at failure of a first-line chemotherapy regimen, while 12 and 8 at failure of second- and third- lines respectively. Demographic characteristics of patients are shown in table 1.

3.2. Plasma ctDNA mutational analysis at inclusion

The mutational analysis of plasma samples at the time of PD was performed using the real time PCR Idylla™ Biocartis system. According to the results of ctDNA mutational analysis at inclusion, two groups of patients were identified. The first included 22 cases (55%) with evidence of RAS mutations in plasma ctDNA samples, with 20 samples bearing the same mutation found at baseline, while 2 being characterized by a different mutation. These patients were classified as "RAS retainers". Among them, eight had failed a first-line regimen, while eight and six had failed a second- and third-line regimen respectively (Tab. 2). Group 2 consisted of 18 patients (45%) in whome no RAS/BRAF mutations were detected in plasma ctDNA samples at PD (RAS/BRAF wt). These patients were classified as "RAS converters". According to lines of therapy, "RAS converters" were observed in 12/20 (60%) patients enrolled at failure of a first-line regimen, while in 4/12 (33%) and in 2/8 (25%) of those who failed a second- or third-line of therapy respectively (Tab. 2). The samples from "RAS converters" patients were further analyzed in order to assess whether the wt RAS state was linked to a true conversion of the mutational state or, otherwise, to an insufficient amount of ctDNA in the sample.

3.3. Defining "true RAS converters" according to NGS analysis

Eighteen ctDNA samples found RAS/BRAF wt at inclusion through real time PCR were further analysed by NGS using the the Oncomine™ Colon cfDNA, with the aim to identify at least one somatic mutation (other than RAS). In 11/18 cases (61%) we were able to identify at least one somatic mutation other than RAS in the ctDNA; these patients were classified as " true RAS converters". Of them, seven patients had failed a first-line regimen, while three and one had failed a second- or third-line regimen respectively. Conversely in 7/18 cases (39%) the analysis in IT-PGM did not reveal any somatic mutation in plasma ctDNA samples (5 at failure of first-line regimens; 1 at failure of second and 1 at failure of third-line). All these were considered not informative, since no somatic mutations other than RAS had been detected in the primary tumor tissue. Therefore, considering the initial patient population, and using NGS to confirm or exclude the presence of ctDNA in the plasma samples, we estimated that a true conversion occurred in 11/40 patients (27.5%) (Tab. 3)

3.4. Defining "true RAS converters" according to methylation test

We further analyzed the 18 wt RAS ctDNA samples with a colon cancer specific methylation assay. 14/18 samples (78%) were found methylated in more than one gene and these patients were classified as "true RAS converters". On the other hand, in 4/18 cases (22%) the methylation test was found negative (not-shedders patients). Three and one of them had failed a first- and second-line respectively. Therefore, using methylation analysis to confirm or exclude the presence of ctDNA in the sample, we estimated that a true RAS conversion occurred in 14/40 patients (35%) (Tab. 4).

3.5. Concordance between NGS and methylation test

NGS and methylation assays gave concordant results in 13/18 cases (72%). In the group of five discordant cases, only one was found positive by NGS but not by the methylation assay. In the remaining four samples, which were classified as not informative by NGS analysis, due to the absence of any somatic mutation other than RAS in the primary tissue, the methylation test was instead found positive. Globally, considering either NGS or methylation as reliable tests to confirm or exclude the presence of ctDNA, the percentage of "true RAS converters" was 37.5% (Tab.5).

3.6. Response to EGFR inhibitors in" true RAS converters"

Among the subgroup of "true RAS converters", as confirmed by NGS and/or methylation analysis (thus being considered for all intents and purposes patients with RAS/BRAF wt disease), ten agreed to enter an experimental study aimed to evaluate the efficacy of EGFR inhibitors in combination with standard chemotherapy. Of them, six received EGFR inhibitors as second-line, while EGFR inhibitors was administered in two patients as a third and in two patients as fourth-line respectively. Median follow-up time was 9 months (range 3-16 months).
Globally considering the whole population, in the group of 20 patients enrolled at failure of first line, six received EGFR inhibitors while 14 did not (median progression free survival (PFS) 6.5 and 5 months respectively). In the group of 20 patients enrolled at failure of second/third-lines, 4 received EGFR inhibitors while 16 did not (median PFS 6.0 and 4.0 respectively) (Tab. 6). Treatment regimens adopted in all patients at the time of PD and relative PFS data are shown in Table S1 (supplementary material).

4. Discussion

An increasing number of studies have confirmed that the analysis of ctDNA in patients with mCRC allows to monitor the temporal heterogeneity of the disease and to personalize treatments [23-25]. Recent evidence has been provided that the clonal evolution of mutant RAS colorectal cancer can lead in some cases to the negative selection of mutant RAS clones, with the appearance of a time window characterized by the prevalence of wt RAS clones in plasma ctDNA [11-13]. From a strictly analytical point of view, however, it must be considered that the absence of RAS mutations as assessed through liquid biopsy might depend on the low amount of ctDNA released in the plasma, with crucial implications for a correct interpretation of the results of liquid biopsy tests. Several liquid biopsy tests are available to date, characterized by different analytical sensitivity [21]. The real time PCR Idylla^{TM} test, which we used in this study to screen RAS mutations in plasma, has an analytical sensitivity $\leq$1% for KRAS mutations of exons 2 and 3 and $\leq$5% for mutations on exon 4. A recent comparison between the Idylla^{TM} test and OncoBEAM performed in plasma samples from patients with mCRC identified a "gray zone" (mutant allelic fraction <1%) in which Idylla showed a reduced

accuracy of RAS mutation detection compared to OncoBEAM [26]. In light of these data, it is imperative to correctly interpret the clearance of RAS mutations in plasma by demonstrating the presence of a sufficient amount of ctDNA, in order to distinguish "non-shedders" patients from those with a true conversion from RAS mutant to RAS wt disease. In the present study we used two different approaches to confirm the presence of ctDNA: NGS and a colon cancer specific methylation test. The use of NGS for liquid biopsy analysis has been limited for a long time by its relatively low sensitivity. In particular, the standard targeted sequencing panels, commonly used for molecular analysis of tissue samples, have a sensitivity of about 1-2% which limits their use for ctDNA tests. In fact, mutations with an allelic frequency of less than 1% are frequently described in patients even with advanced neoplasms. The development of new NGS techniques with increased sensitivity has allowed this limit to be overcome [27]. Despite the increased sensitivity, monitoring other somatic mutations previously detected in the primary tumor tissue is not always a feasible approach, if one considers that roughly 30% of RAS mutant primary tumors do not bear other somatic mutations [19]. Furthermore, the mutational analysis of ctDNA with NGS must take into consideration the possibility of false positives related to the phenomenon of clonal hematopoiesis [28], being the presence of germline variants derived from normal cells a further source of confusion in the interpretation of the data. The frequency of mutations associated with clonal hematopoiesis increases with age and the possibility of identifying them with an NGS test on cfDNA depends on the sensitivity of the technology used. Similarly, the analysis of metastatic sites to assess RAS mutational status at the time of disease progression is feasible in an absolute minority of patients with surgically accessible metastases. In our hands the methylation test was found more suitable for discriminating non-shedders patients from true converters. The advantages of the methylation assay compared to the NGS analysis are mainly related to the unnecessary knowledge of the somatic mutations present on the primary tumor tissue, particularly relevant in those cases with tissue biopsies lacking somatic mutations other than RAS. In fact, 4 plasma samples, in which the wt RAS status in plasma could not be confirmed by the presence of other somatic mutations neither in plasma nor in the primary tumor tissues (thus considered not informative), were instead found “true RAS converters” according to the methylation test. Although some studies are now investigating the biological phenomenon of RAS conversion in patients with metastatic colorectal tumors, interpretation of data is still confusing. Moati et reported that only 6.6% of mutant RAS mCRC patients convert to RAS wt in plasma at the time of disease progression, using two cancer specific methylated genes (WIF1 and NPY) as a confirmation of ctDNA presence [29]. Nevertheless, evidence has been provided that NPY is strongly methylated in normal blood samples, and that WIF1 is just as little specific in discriminating normal from tumoral colic tissues [22]. In a second study 119 patients with mutant RAS mCRC were monitored with liquid biopsy every 8 weeks until disease progression. Disappearance of RAS mutations in plasma was defined in conjunction with at least one genetic mutation detectable by NGS analysis. This study reported RAS conversions in 30% of cases [30].

Bouchada et al, using Massarray Ultraseek technology, reported a conversion rate of 56% (ref). Patients with evidence of absence of RAS mutations at the time of disease progression were treated with EGFR inhibitors, with an objective response rate of 50% [31]. Klein Scory et al monitored plasma RAS mutations in 12 patients with primary diagnosis of RAS mutant colorectal cancer undergoing first-line treatment using droplet PCR and Beaming reporting in 91% of cases a RAS mutation clearance which occurred very early in course of treatment. The concentration of ctDNA in plasma was assessed using the WIF-1 gene methylation assay, and the persistence of methylated sequences was considered as a surrogate for plasma ctDNA release [12]. Authors conclude that the RAS mutational load in blood is a biomarker of negative clonal selection of RAS-mutated clones, since patients initially converted from RAS mutated to RAS wt remained RAS wt at the time of

progressive disease. Conversely, we have previously analyzed plasma samples of patients with mutant RAS tumors at different time-points during treatment using the 5-gene methylation panel to confirm or exclude the presence of ctDNA. In contrast to what reported by Klein-Scory, we failed to find methylated sequences in all plasma samples collected 4 months after starting treatments, thus suggesting that the detection of wt RAS status in plasma early in course of therapy more probably reflects the absence of ctDNA in the samples. Conversely, at the first and second progressions, 63% and 50% of the plasma wt RAS samples were found to be methylated, respectively [18]. Similarly, Sunakawa et al reported a RAS mutation conversion rate of 76% after 4 cycles of chemotherapy, interpreting the decrease of RAS mutational load as an early predictor of response [32]. The discrepancy between our data and those of Klein-Scory could again be explained by a possibly lower specificity of the methylation test used by Klein-Scory to confirm the presence of ctDNA.
In conclusion, RAS conversion rate in primary mutant colorectal cancers is extremely variable according to studies, ranging between 6% and 70%, strictly dependent on the timing of blood collection and on the liquid biopsy method used. Some studies have demonstrated, although in different therapeutic lines, the potential efficacy of EGFR inhibitors in this group of patients [11,12,31]. Although the mixed lines of therapy and the small numbers of treated patients did not allow us to draw any statistically significant conclusion, in our series we observed a trend toward a better PFS in patients who received anti-EGFR as second or subsequent treatment lines compared to those who did not. Given the paucity of targeted treatments available in patients with mutant RAS colorectal cancers, and the consequent poor prognosis of this patient group compared to those with RAS wt disease, the possibility of including EGFR inhibitors in any line of therapy seems an attractive hypothesis. Some phase II trial are currently ongoing to investigate whether patients with RAS-mutant mCRC at diagnosis will have a PFS benefit from addition of EGFR inhibitors to first-line therapy after RAS-mutation status has changed to wt [11,12].

Declaration of competing interest

The authors declare no conflicts of interest.

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