Evaluation of ct-DNA and mutational status in liquid biopsy of patients with metastatic colorectal cancer

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Abstract. Liquid biopsy has become an increasingly important tool in clinical oncology due to the utility of circulating tumor DNA (ct-DNA). Plasma ct-DNA levels reflect the tumor burden of patients and provide valuable information for personalized treatment strategies. Colorectal cancer (CRC) is among the most frequently diagnosed malignancies worldwide and remains a leading cause of cancer-related mortality. The implementation of personalized therapy can notably improve clinical outcomes in these patients. The present study aimed to evaluate ct-DNA in patients with metastatic CRC undergoing therapy at the Department of Medical Oncology, University Hospital of Ioannina (Ioannina, Greece), using next-generation sequencing (NGS) to identify tumor mutations in both tissue and cell-free plasma DNA. A total of 45 patients newly diagnosed with metastatic CRC were enrolled in the current study. ct-DNA evaluation was performed at diagnosis, and both tissue and plasma samples were analyzed using NGS to assess the mutational profile. The most frequent mutation identified was in *KRAS*. A high level of concordance was observed between tissue and plasma samples regarding ct-DNA detection and mutational status. Sequential ct-DNA analysis during therapy revealed dynamic changes in mutation profiles over the course of treatment. In conclusion, liquid biopsy offers marked clinical value as a minimally invasive tool for personalized therapy in metastatic CRC. NGS-based liquid biopsy enables real-time evaluation of ct-DNA and mutation status during treatment, supporting therapeutic decision-making and disease monitoring.

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Introduction

Over the last decade, liquid biopsy have attracted increasing attention in modern oncology due to its potential clinical applications. Circulating tumor cells, circulating tumor nucleic acids [such as circulating tumor DNA (ct-DNA) and circulating tumor RNA], exosomes, and tumor proteins are considered as possible biomarkers for characterizing the molecular characteristics of malignant tumors, thus providing insights into both the genetic and phenotypic features of cancers, eventually facilitating the development of personalized medicine (1,2).

ct-DNA is a fraction of total cell-free DNA found in blood plasma. Under normal conditions, plasma DNA originates from hematopoietic cells and is present at low concentrations in healthy individuals. DNA enters the bloodstream through several processes, such as cell death, necrosis, or apoptosis (3). However, inflammation, intense exercise, or severe stress can also enhance the content of circulating free DNA. In patients with cancer, ct-DNA fragments encompass the same somatic mutations as those found in the primary tumor. On average, ct-DNA fragments are ~143-145 base pairs in length-shorter than normal cell-free DNA-and exhibit a short half-life of ~2 h. The liver serves as the primary site for ct-DNA clearance from the circulation (3,4).

It has been reported that plasma ct-DNA levels can be associated with a patient's tumor burden, with advanced cancers typically displaying higher concentrations of ct-DNA in the bloodstream. The amount of ct-DNA released into the circulation also depends on several factors, including tumor location, cell proliferation, and necrosis. Bettegowda *et al* (5) demonstrated that patients with localized disease or primary central nervous system tumors rarely exhibited detectable ct-DNA levels, while those with metastatic colon, pancreatic, bladder, or ovarian cancers commonly did. Furthermore, the short half-life of ct-DNA can also enable real-time monitoring of tumor clonal heterogeneity during the disease course.

Pre-analytical and analytical variables can significantly affect ct-DNA analysis. Factors such as the type of blood collection tubes used, volume of plasma processed, storage conditions, and specimen handling can markedly affect the accuracy of ct-DNA results. The DNA extraction method

should therefore be carefully considered to ensure optimal reproducibility (6,7). Beyond technical aspects, several clinical and biological factors, including the timing of sample collection, disease stage, prior or ongoing treatments, and coexisting medical conditions, can also affect the proportion of detectable ct-DNA fraction in plasma. However, the application of highly sensitive analytical techniques, such as next-generation sequencing (NGS) or digital PCR, has significantly improved the detection threshold for ct-DNA in patients with cancer (8,9).

ct-DNA serves as a liquid biopsy biomarker with unique advantages that help overcome the limitations of conventional 'gold standard' tissue biopsies. Blood collection is simple, minimally invasive, and safe, posing minimal risk compared with interventional tissue sampling (10). Additionally, rabid analysis of tumor-derived DNA can offer a minimally invasive method, particularly in cases where conventional biopsy is difficult to perform, unsafe, or when tissue samples are insufficient. The ability to obtain serial blood samples also eliminates the need for repeated tissue biopsies, thus providing dynamic insight into the molecular profile of the tumor over time. This approach enables real-time monitoring of spatial and temporal tumor heterogeneity, facilitates the assessment of treatment response, and aids the early detection of resistant clones, ultimately supporting more precise clinical decision-making (11,12).

Colorectal cancer (CRC) is one of the most prevalent types of cancer worldwide and remains a leading cause of cancer-related mortality (13). The therapeutic landscape of metastatic CRC has evolved significantly over the past decade, with the introduction of targeted therapies, immunotherapies, and advanced chemotherapy regimens, alongside continuous efforts to improve overall survival. According to current clinical guidelines, the assessment of microsatellite instability (MSI) and *RAS* and *BRAF* mutations is a critical step prior therapy initiation. The detection of *RAS* or *BRAF* mutations excludes the use of anti-epidermal growth factor receptor (EGFR)-targeted therapy, directing patients instead toward antiangiogenic and chemotherapeutic combinations. For patients initially treated with chemotherapy plus anti-EGFR therapy, switching to alternative regimens upon disease progression is recommended (14).

However, CRC is a biologically heterogeneous disease showing both spatial and temporal diversity, and acquired anti-EGFR resistance mechanisms can emerge over time. In this context, liquid biopsy represents a valuable tool for capturing the molecular characteristics of metastatic CRC throughout the course of treatment, thus guiding therapeutic decision-making (15). Recently, the ESMO Precision Medicine Working Group issued recommendations supporting the integration of ct-DNA analysis into clinical practice, identifying CRC as one of the major areas of application (16).

The present study aimed to assess ct-DNA in patients with metastatic CRC undergoing therapy, using NGS to identify tumor-related mutations in both tissue samples and cell-free plasma DNA.

Materials and methods

Patients. In the current study, a total of 45 patients who attended the University Hospital of Ioannina (Ioannina, Greece) between September 2018 and November 2022 were enrolled. All participants were required to have a new diagnosis of metastatic CRC and an adequate tumor tissue sample available for molecular profiling. In addition, blood samples were collected from patients at multiple time points during the course of treatment. Specifically, blood collection for ct-DNA isolation and analysis was performed at diagnosis (prior to therapy initiation) and at the time of first and second disease progression. Both tumor tissues and plasma samples were subjected to NGS for comprehensive mutational profiles. All patients provided written informed consent prior to enrollment and the study protocol was approved by the Ethics Committee of the University Hospital of Ioannina (approval no. 11/18-04-2018).

Specifically, the inclusion and exclusion criteria were as follows. Eligible participants were adults aged 18 years or older with a new diagnosis of metastatic colorectal adenocarcinoma who had adequate FFPE tumor tissue available for molecular profiling, were able to provide serial blood samples at baseline and during follow-up, and provided written informed consent for participation and molecular testing. Patients were excluded if they had received prior systemic treatment for metastatic disease before baseline sampling, lacked sufficient tumor tissue for NGS analysis, were unable to provide the required blood samples for ctDNA testing, or had a coexisting malignancy or medical condition that could interfere with proper sample collection or reliable NGS analysis.

Cell-free total nucleic acids (cfTNA) isolation. Peripheral blood samples were collected into Cell-Free DNA BCT tubes (Streck LLC.), particularly designed to preserve cfTNA. Plasma was obtained through two sequential centrifugations at 447 x g for 10 min at 4˚C to ensure complete removal of cellular components. cfTNA was then extracted from 2 ml plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Inc.), according to the manufacturer's instructions. To minimize pre-analytical variability, all blood samples were processed within 2 h of venipuncture. Plasma aliquots were stored at -80˚C, and repeated freeze-thaw cycles were avoided to preserve cfTNA integrity.

Tissue selection and DNA extraction. Formalin-fixed, paraffin-embedded (FFPE) tumor biopsy sections were stained with hematoxylin and eosin to ensure that the tumor cell content exceeded 75% whenever feasible. A pathologist identified and marked the representative tumor areas for DNA extraction. Genomic DNA was then isolated from 10 μm-thick unstained FFPE sections using the QIAamp FFPE Tissue Kit (Qiagen, Inc.). DNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). To ensure consistency, DNA quality and yield were further verified by Qubit fluorometric analysis prior library preparation.

Preparation of NGS libraries. For the analysis of FFPE-derived DNA samples, a custom Ion AmpliSeq panel was employed, based on the Ion AmpliSeq Colon and Lung Cancer Research Panel v2 (Thermo Fisher Scientific, Inc.). This panel was modified to include one additional amplicon targeting exon 14 skipping mutations in the *MET* gene and

two additional amplicons covering exons 2 and 3 of the *HRAS* gene. Fusion RNA transcript analysis was performed using the Ion AmpliSeq™ RNA Fusion Lung Cancer Research Panel (Thermo Fisher Scientific, Inc.). A detailed list of the 23 targeted genes is available upon request. The analyzed genes included *AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH, NRAS, PIK3CA, PTEN, SMAD4, STK11, TP53,* and *HRAS*. The DNA concentrations from FFPE samples were measured using the Qubit™ 2.0 Fluorometer and the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.). Libraries were prepared from 10 ng of FFPE-derived DNA or 6 μl of ct-DNA using the Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Amplicon amplification was achieved with the Ion AmpliSeq™ HiFi Master Mix (Thermo Fisher Scientific, Inc.), and the amplified products were digested with FUPA reagent followed by barcoding with the IonCode™ Barcode Adapters 1-384 Kit (Thermo Fisher Scientific, Inc.). Libraries were purified using the Agencourt AMPure XP PCR purification system (Beckman Coulter, Inc.).

For total cfTNA analysis, the Oncomine™ Pan-Cancer Cell-Free Assay (Thermo Fisher Scientific, Inc.) was utilized. This assay incorporates unique molecular identifiers (UMIs) in the form of random molecular tags to label each molecule prior library amplification, thus enabling the distinction of true variants from random sequencing errors, thereby enhancing accuracy and minimizing false-positive results. The assay targeted hotspot mutations, including single-nucleotide variants (SNVs) and small insertions or deletions, in genes such as *AKT1, ALK, AR, ARAF, BRAF, CHEK2, CTNNB1, DDR2, EGFR, ERBB2, ERBB3, ESR1, FGFR1-4, FLT3, GNA11, GNAQ, GNAS, HRAS, IDH1, IDH2, KIT, KRAS, MAP2K1, MAP2K2, MET, MTOR, NRAS, NTRK1, NTRK3, PDGFRA, PIK3CA, RAF1, RET, ROS1, SF3B1, SMAD4,* and *SMO*. Copy number variations were also assessed in *CCND1-3, CDK4, CDK6, EGFR, ERBB2, FGFR1-3, MET,* and *MYC*, and RNA fusions involving 12 key driver genes, including *ALK, BRAF, ERG, ETV1, FGFR1-3, MET, NTRK1, NTRK3, RET,* and *ROS1,* were also evaluated. The analytical sensitivity of the cfTNA assay was validated at ~0.1-0.5% mutant allele fraction (MAF) for SNVs/indels, ~1% for gene fusions, and ≥1.4-2-fold change for copy number variation (CNV) detection. For FFPE-derived DNA, the Ion AmpliSeq workflow reliably detected mutations with a limit of detection of ~5% MAF, applying a minimum coverage threshold of 500x for confident variant calling. cfTNA concentrations were quantified using the Qubit™ 2.0 Fluorometer and the Qubit™ ssDNA Assay Kit (Thermo Fisher Scientific, Inc.), while libraries were prepared according to the manufacturer's instructions. Briefly, reverse transcription of cfTNA was carried out using the SuperScript™ VILO™ Master Mix. Subsequently, the resulting cDNA was subjected to a two-cycle PCR to attach random sequence tags and A/P1 adaptors to both fragment ends, followed by purification using the Agencourt AMPure XP PCR system (Beckman Coulter, Inc.). An additional 18-cycle PCR was then performed to amplify the tagged fragments and introduce a unique barcode for each sample using the Tag Sequencing Barcode Set (Thermo Fisher Scientific, Inc.). Purification and size selection of the barcoded libraries were carried out with Agencourt AMPure XP beads, and library concentrations were subsequently quantified by real-time PCR using the Ion Library TaqMan® Quantitation Kit (Thermo Fisher Scientific, Inc.).

For clonal amplification, 100 pmol of DNA or cfTNA libraries were pooled and amplified on Ion Sphere™ Particles (ISPs) via emulsion PCR using the Ion OneTouch™ 2 instrument and the Ion 540™ Kit-OT2, following the manufacturer's guidelines. Quality control was then performed with the Ion Sphere™ Quality Control Kit to ensure that ~10-30% of ISPs were template-positive. Enrichment of template-positive ISPs was carried out on the Ion OneTouch™ ES instrument. The enriched particles were then loaded onto an Ion 540™ Chip and sequenced on the Ion GeneStudio™ S5 Prime System (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

Analysis of NGS data. NGS data analysis was performed using Ion Reporter™ software version 5.10.1.0 integrated with Torrent Suite™ version 5.10.1 (Thermo Fisher Scientific, Inc.). The sequencing results were subsequently reviewed and further analyzed using Sequence Pilot (version 4.3.0; JSI Medical Systems GmbH). Coverage analysis was conducted with the Coverage Analysis plug-in (version 5.0.4.0), providing quality metrics for each library within the sequencing run. For FFPE DNA libraries, CNV analysis was carried out using Ion Reporter™ software, which determines CNVs based on relative copy number compared with a reference control sample. CNVs were assigned confidence scores, with values >10 indicating high-confidence amplifications, serving as the threshold for defining copy number gains. For cfTNA samples, mutation detection, RNA fusion identification, and CNV analysis were conducted according to the manufacturer's workflow using Ion Reporter™ Oncomine TagSeq Pan-Cancer Liquid Biopsy v2.1-Single Sample software (Thermo Fisher Scientific, Inc.). Furthermore, the distribution of MAFs in both plasma and tissue samples was also evaluated. All sequencing runs incorporated internal quality controls, including coverage metrics and spike-in control samples, to ensure reproducibility and instrument stability across all experiments.

Statistical analysis. The primary objective of the current study was to evaluate the concordance between mutation detection in tumor tissue DNA and cell-free plasma DNA. To accomplish this, tumor mutations were identified and compared in both sample types, and Cohen's κ statistic was then performed to assess the level of agreement. More particularly, all statistical analyses were conducted using the R programming language (R Core Team: R: A Language and Environment for Statistical Computing. Vienna, Austria: Foundation for Statistical Computing; https://www.r-project.org/). Continuous variables are expressed as the mean ± standard deviation and median with interquartile range (IQR), while categorical variables are presented as counts (n) and percentages (%). Categorical variables were compared using either the χ^2 test or Fisher's exact test, as appropriate, whereas paired nominal data, defined as categorical measurements obtained from the same patient in tumor tissue and plasma samples, were analyzed using the McNemar test. The normality of continuous variables was assessed by Kolmogorov-Smirnov test. Based on

data distribution, comparisons of continuous data between independent (unpaired) groups were performed using the Mann-Whitney U test for non-normally distributed data. KRAS MAF values did not follow a normal distribution; therefore, comparisons between tissue and plasma samples were conducted using the Mann-Whitney U test. Correlations between continuous variables were evaluated using Pearson's or Spearman's rank correlation coefficients, as appropriate. Sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and overall agreement were estimated with 95% confidence intervals (CIs) using binomial distribution. Agreement represents the degree of concordance between the two methods, and Cohen's κ was employed to quantify intermethod reliability. Two-tailed P-values and 95% CIs calculated using Fisher's r-to-z transformation. $P<0.05$ was considered to indicate a statistically significant difference. For Spearman's rank correlation analyses, two-tailed P-values and 95% confidence intervals were calculated using Fisher's r-to-z transformation, with standard error estimated according to the method of Fieller, Hartley and Pearson.

Results

Patient characteristics. Among the 45 patients enrolled in this study, 18 were female and 27 were male, all diagnosed with metastatic colorectal adenocarcinoma. The primary tumor was located in the left colon in 32 patients, the right colon in nine patients, and the transverse colon in three patients, while one patient had an unknown primary tumor site. The most common metastatic sites were the liver, lungs, and peritoneum. A total of eight patients had previously received adjuvant chemotherapy with 5-fluorouracil, either alone or in combination with oxaliplatin, following an earlier diagnosis of stage III CRC. The remaining patients presented with *de novo* metastatic disease at initial diagnosis. MSI testing was performed in a total of 33 patients. Among them, 2 patients showed loss of mismatch repair protein expression, consistent with MSI, while the remaining 31 patients had microsatellite-stable (MSS) tumors. All patients were treated with first-line therapy for metastatic CRC, according to the current clinical guidelines and the treating physician's discretion. More specifically, 9 patients received first-line therapy with mFOLFOX6 combined with an anti-EGFR agent, seven received mFOLFOX6 plus bevacizumab, and one patient was treated with mFOLFOX6 alone. Fourteen patients received XELOX plus bevacizumab, one received capecitabine plus bevacizumab, and one received XELOX alone. In addition, 3 patients were treated with capecitabine monotherapy, 4 received FOLFIRI plus an anti-EGFR agent, 2 received FOLFIRI plus bevacizumab, 2 were treated with FOLFOXIRI plus bevacizumab, and 1 received FOLFIRI alone. The demographic and clinical characteristics of the study population are summarized in Table SI.

Tissue NGS results. Among the 45 tissue samples analyzed by NGS, 24 samples harbored *KRAS* mutations. The most common variants detected were *KRAS* c.35G>A (p.G12D) in 6 samples, *KRAS* c.38G>A (p.G13D) in 6 samples, *KRAS* c.35G>T (p.G12V) in 3 samples, and *KRAS* c.34G>A (p.G12S) in 3 samples. Less common variants included *KRAS* c.64C>A (p.Q22K), *KRAS* c.436G>A (p.A146T), *KRAS* c.183A>C (p.Q61H), and *KRAS* Q61R in 1 patient each, and *KRAS* c.35G>C (p.G12A) in 2 patients. In addition, 2 patients carried *NRAS* mutations, and more specifically *NRAS* c.38G>T (p.G13V) and *NRAS* c.181C>A (p.Q61K). *BRAF* V600E mutations were detected in three patients, while 1 patient carried a *BRAF* G466E mutation. Notably, *KRAS*, *NRAS*, and *BRAF* mutations were mutually exclusive in all tissue samples. During tissue profiling with NGS, among the remaining genes included in the sequencing panel, 27 patients carried *TP53* mutations, 6 *PIK3CA* mutations, 1 patient harbored an *APC* mutation, 2 *SMAD4* mutations, and two *FBXW7* mutations. Overall, mutations in genes other than *KRAS*, *NRAS*, and *BRAF* were identified in 35 of the 45 tissue samples analyzed.

Baseline plasma ct-DNA results. Among the 45 baseline plasma samples analyzed by NGS, 20 harbored *KRAS* mutations, including *KRAS* c.35G>A (p.G12D) mutation in 5 samples, *KRAS* c.38G>A (p.G13D) in 6 samples, *KRAS* c.35G>T (p.G12V) in 2 samples, *KRAS* c.34G>A (p.G12S) in 3 samples, *KRAS* c.35G>C (p.G12A) in 1 sample, *KRAS* c.183A>C (p.Q61H) in 2 samples, while 1 sample carried a *KRAS* c.436G>A (p.A146T) mutation. In addition, 2 patients carried *NRAS* mutations, specifically *NRAS* c.38G>T (p.G13V) and *NRAS* c.181C>A (p.Q61K). Furthermore, *BRAF* V600E mutations were identified in 3 samples. As observed in tissue samples, *KRAS*, *NRAS*, and *BRAF* mutations were mutually exclusive in plasma samples. Beyond the aforementioned mutations detected in plasma samples after ct-DNA isolation, additional changes were identified in other genes included in the NGS panel. Specifically, 17 samples carried *TP53* mutations, four harbored *PIK3CA* mutations, two had *APC* mutations, and *PTEN*, *MET*, *FBXW7*, and *AKT* mutations were identified in one sample each. Overall, 24 plasma samples tested positive for alterations in genes other than *KRAS*, *NRAS*, and *BRAF*.

Following first-line treatment and at first disease progression, blood samples were again collected from 33 patients and processed for ct-DNA isolation and mutation analysis. Among them, 10 patients harbored *KRAS* mutations, including *KRAS* c.35G>A (p.G12D) in four patients, *KRAS* c.35G>T (p.G12V) in 2 patients, *KRAS* c.38G>A (p.G13D) in 2 patients, *KRAS* Q61R in one patient, while 1 patient carried co-occurring *KRAS* c.38G>A (p.G13D) and *KRAS* c.34G>A (p.G12S) mutations. *NRAS* mutations were detected in 2 samples, specifically *NRAS* c.38G>T (p.G13V) and *NRAS* c.183A>T (p.Q61H). In addition, 3 samples harbored a *BRAF* V600E mutation. No samples showed concurrent *KRAS*, *NRAS*, and *BRAF* mutations. At first disease progression, additional mutations were also identified in other genes. Particularly, *APC* mutations were found in four samples, *TP53* mutations in 13 samples, and *SMAD4* mutations in two samples. Furthermore, copy number amplifications were detected in *EGFR* (one sample) and *FGFR1* (one sample). Overall, among the 33 available samples at disease progression, 18 carried mutations in genes other than *KRAS*, *NRAS*, and *BRAF*.

At second disease progression, 2 blood samples were collected. Both samples harbored *KRAS* c.38G>A (p.G13D) mutations, while remaining wild-type for *NRAS* and *BRAF*. Among them, one sample carried an *APC* mutation, and the other harbored a *TP53* mutation.

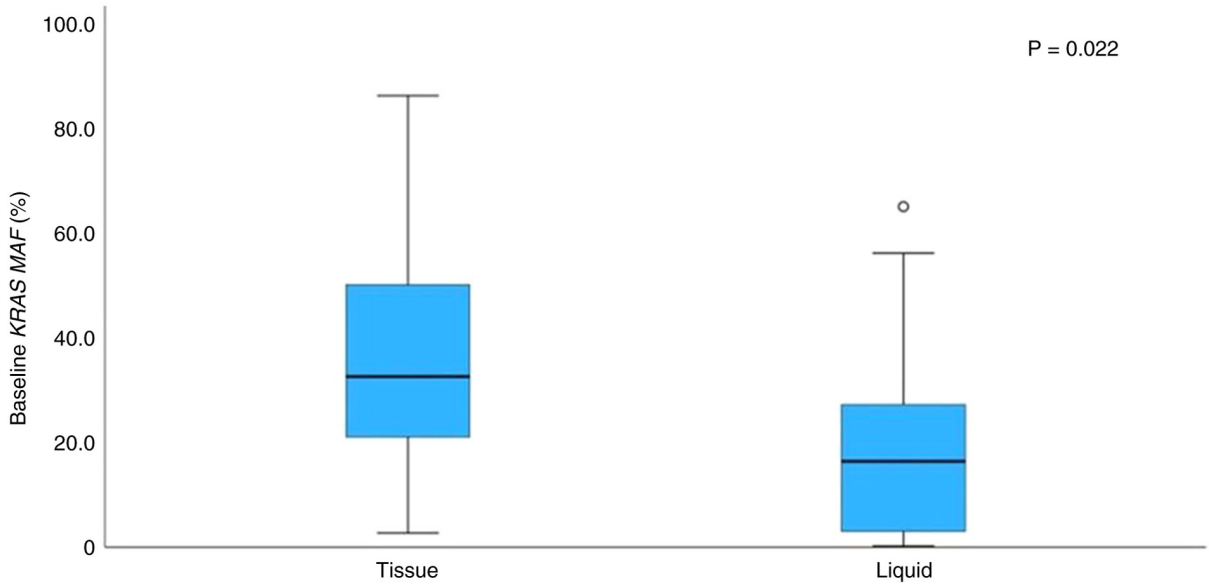

Figure 1. Baseline *KRAS* MAF in both tissue and liquid samples (P=0.022). MAF, mutant allele fraction.

Concordance between tissue and baseline plasma ct-DNA. The results of the statistical analysis demonstrated a high level of concordance between each mutation tested. More specifically, for *KRAS* mutations, the overall agreement rate between tissue and plasma samples was 87%, with a PPV of 95% and a NPV of 80.8% [Cohen's κ=0.74 (0.55-0.93); Se=79.2%; Sp=95.5%]. The combined analysis of *KRAS* and *NRAS* mutations also showed an agreement rate of 87%, with PPV=95.5% and NPV=79.2% [Cohen's κ=0.74 (0.55-.93); Se=80.8%; Sp=95%). For *BRAF* mutations, concordance was even higher, reaching 97.8%, with PPV=100% and NPV=97.8% [Cohen's κ=0.85 (0.55-1.00); Se=75%; Sp=100%]. For other, less frequent mutations, a positive agreement of 67.4% was also observed, with PPV=40.9% and NPV=67.4% [Cohen's κ=0.33 (0.09-0.57); Se=62.9%; Sp=81.8%). The McNemar test verified these findings, showing no statistically significant differences in the detection of *KRAS*, *NRAS*, and *BRAF* mutations between tissue and plasma samples (P>0.05; Table SII).

Regarding *KRAS*, the median MAF in tissue samples was significantly higher compared with plasma samples (Mann-Whitney test, P=0.022; Fig. 1). However, Spearman's correlation analysis showed no significant association between tissue and plasma *KRAS* MAF values (Spearman's ρ=0.25; P=0.313). In several cases, MAF values were higher in plasma compared with tissues, and vice versa. The above data are summarized in Table SIII.

Change in mutational status with serial ct-DNA monitoring. The assessment of changes in mutational status during disease progression is of great importance. At progression, one patient who was initially *KRAS/NRAS* wild-type developed a novel *KRAS/NRAS* mutation, while seven patients who harbored *KRAS/NRAS* mutations at baseline lost these mutations at progression. Similar findings were observed in plasma samples. More particularly, two patients acquired new *KRAS/NRAS* mutations at progression, while six patients lost their baseline *KRAS/NRAS* mutations during progression. By contrast, the *BRAF* mutational status remained largely stable throughout the disease course. All patients who were *BRAF* wild-type at baseline maintained their wild-type status during progression, while only one patient with a *BRAF* mutation at baseline reverted to wild-type status during progression. However, no changes in *BRAF* mutational status were detected in liquid biopsy samples between baseline and progression. Among other genes included in the sequencing panel, six patients developed new mutations at progression compared with baseline tissue samples, while 12 patients lost previously detected mutations. Consistently, in baseline plasma samples, 10 patients developed new mutations, and eight patients lost prior mutations during progression. The detailed mutational status for *KRAS*, *BRAF*, and other genes is summarized in Table SIV. Overall, 62.5% of patients with *KRAS* or *NRAS* mutations at baseline retained these mutations during progression, whereas 37.5% lost them. Conversely, 11.8% of patients who were wild-type *KRAS/NRAS* at baseline developed new mutations at progression, while 88.2% remained wild-type (Fig. 2).

Discussion

Understanding the molecular profile of cancer is essential for guiding therapy decisions, personalizing treatment strategies, and ultimately improving patient outcomes. However, tumor tissue samples are commonly insufficient for performing all required molecular analyses and are often difficult to obtain for re-evaluation during disease progression, particularly in cases requiring fine-needle aspiration biopsies. Therefore, a faster and less invasive technique is urgently needed. In this context, detecting and analyzing ct-DNA and tumor mutational status through liquid biopsy represents a promising approach (5,17).

ct-DNA analysis can enable the assessment of molecular heterogeneity and provide novel insights into the overall mutational landscape of the disease. It further allows real-time monitoring of tumor dynamics, reflecting the evolving nature of cancer progression. Since the molecular profile of cancer can change over time, liquid biopsy offers a convenient, accurate and repeatable alternative to repeated conventional

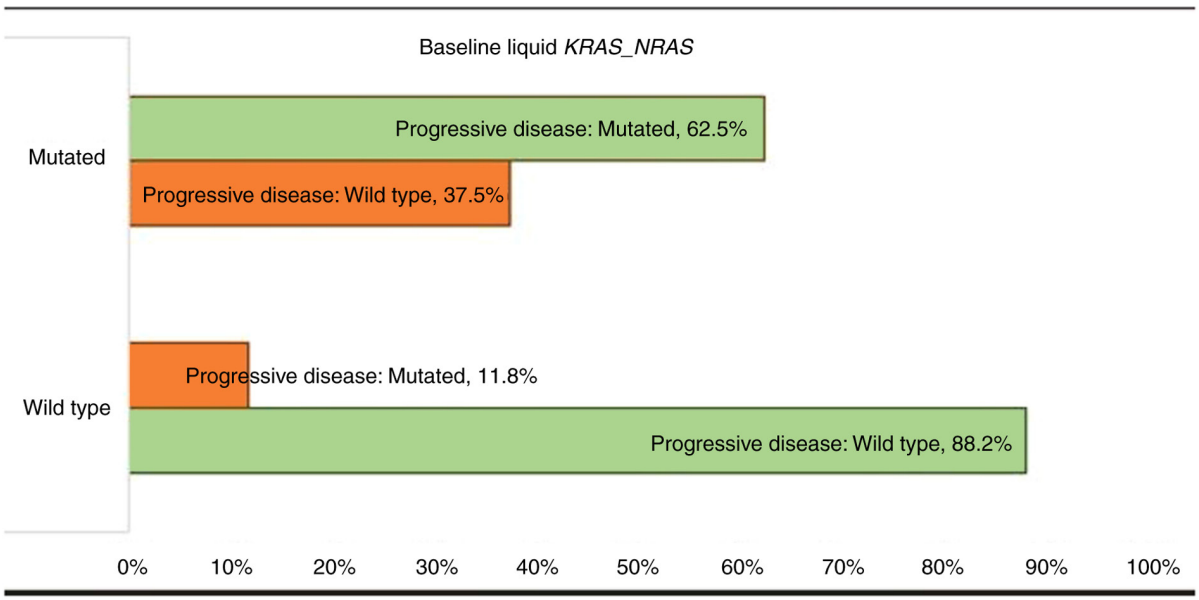

Figure 2. Mutational changes in *KRAS* and *NRAS* during progressive disease.

tissue biopsies. Furthermore, ct-DNA serves as a valuable prognostic biomarker, thus aiding in the early detection of disease progression and in identifying patients at higher risk of poor outcomes (5,18).

Tie *et al* (19) demonstrated that ct-DNA could serve as a strong indicator for predicting recurrence after surgery, independent of adjuvant treatment. In the context of targeted therapy, integrating liquid biopsy into clinical practice is crucial for detecting ct-DNA, eventually allowing the early identification of resistance-related mutations and timely therapeutic adjustments. Liquid biopsies also play a key role in real-time evaluation of resistance mechanisms, thus improving prognostic assessment and identifying patients at higher risk of recurrence. Importantly, it has been reported that ct-DNA levels are associated with tumor burden, thereby offering clinicians a more comprehensive understanding of disease progression and treatment efficacy (17,20).

Conversely, Siravegna *et al* (21) emphasized the challenges associated with liquid biopsy, including the need for standardization of blood collection and processing procedures to maintain sample stability at room temperature and to minimize pre-analytical variability. Ongoing efforts aim to develop standardized methods for ct-DNA quantification, optimizing isolation protocols to enhance ct-DNA yield, and improving the sensitivity of ct-DNA detection. The aforementioned advancements are particularly significant for rare molecular alterations, which are commonly critical for the early detection of drug resistance.

Herein, ct-DNA and mutational profiling was performed in 45 patients with metastatic CRC using both tissue and plasma samples. The most commonly identified mutations involved *KRAS*, *NRAS*, and *BRAF*, along with additional mutations in several other genes. When *KRAS*, *NRAS*, and *BRAF* mutations were excluded, 78% of samples carried mutations in other genes. The analysis revealed strong concordance between mutations detected in tissue and plasma samples, thus supporting the increased clinical feasibility of liquid biopsy during therapy. Furthermore, dynamic changes in mutational status throughout disease progression were detected, with some wild-type genes acquiring mutations and others reverting to wild type. Therefore, liquid biopsy could provide an accessible and reliable alternative for genetic testing, thus enabling more informed and timely treatment decisions.

In our cohort, among the 26 patients with KRAS/NRAS mutations detected in baseline tumor tissue, 62.5% (15/24) of those with available serial plasma samples retained their mutation at first progression, whereas 37.5% (9/24) demonstrated complete clearance in circulating DNA. Conversely, among baseline KRAS/NRAS wild-type patients, 11.8% (2/17) developed a new RAS mutation at progression. Descriptively, patients with persistent KRAS/NRAS mutations were more likely to experience radiologic progression at the corresponding time point, while those with mutation clearance more frequently achieved longer intervals of disease control. Although the sample size limits definitive statistical conclusions, these numerical trends support the potential prognostic value of ct-DNA mutation dynamics.

Particularly, *KRAS*, *NRAS*, and *BRAF* are key components of the rat sarcoma viral oncogene homolog (RAS)/rapidly accelerated fibrosarcoma/mitogen-activated protein kinase (MAPK) signaling pathway, which is known to regulate cell growth, proliferation, and survival. Mutations in *KRAS* and *NRAS*, both encoding members of the RAS family of proteins, can result in constitutive activation of downstream signaling cascades. Consistently, mutations in *BRAF*, a serine/threonine-protein kinase acting further downstream, drive aberrant pathway activation. These molecular alterations can collectively promote uncontrolled tumor cell proliferation, contribute to oncogenesis, and play a critical role in therapeutic resistance. Clinically, *KRAS* and *NRAS* mutations are well-established predictive biomarkers of resistance to anti-EGFR therapies, whereas the *BRAF* V600E mutation is associated with aggressive tumor biology, poor prognosis, and limited responsiveness to standard chemotherapy. Understanding the biological

functions of these genes is therefore essential for guiding personalized therapy, interpreting the current findings, and reinforcing the utility of liquid biopsy in detecting these alterations to enable timely treatment decisions (6,15,18,22).

An important finding of the present study was the presence of mutational shifts between baseline tumor profiles and subsequent plasma ct-DNA analyses during disease progression. These shifts are clinically relevant, as they often reflect adaptive mechanisms of therapeutic resistance. For example, the emergence of *KRAS* or *NRAS* mutations following treatment with anti-EGFR antibodies has been well-documented and represents a classic mechanism of on-target resistance, rendering continued EGFR blockade ineffective. Similarly, the acquisition of *MET* amplification or *HER2* alterations can act as bypass mechanisms, maintaining MAPK or phosphatidylinositol 3-kinase signaling activation despite upstream inhibition.

From a therapeutic perspective, the detection of such resistance-associated alterations in plasma underscores the potential of liquid biopsy as a dynamic tool to guide treatment adaptation. Patients who develop *RAS* mutations during anti-EGFR therapy could benefit from switching to anti-vascular endothelial growth factor-based regimens, while identification of *MET* amplification or *HER2* overexpression could promote the use of targeted inhibitors against these factors. Furthermore, the detection of acquired gene fusions or rare activating mutations could facilitate patient enrollment in biomarker-driven clinical trials investigating novel agents against emerging resistance mechanisms.

Equally important was the observation of mutational clearance or persistence in plasma during therapy. Clearance of tumor-specific variants could indicate a favorable treatment response and improved prognosis, whereas persistence or re-emergence could suggest minimal residual disease and early relapse, often preceding radiographic evidence of progression. These findings underscored the significant value of serial ct-DNA monitoring into clinical practice-not only for identifying resistance but also for prognostic assessment and early therapeutic intervention. In the current cohort, preliminary descriptive analysis suggested that patients with persistent *KRAS*/*NRAS* mutations were more likely to experience disease progression, while those demonstrating clearance achieved longer disease control. Although the limited sample size could constrain the statistical power of these associations, the observed trends could reinforce the prognostic relevance of ct-DNA dynamics.

Although this study was not designed or powered to draw definitive conclusions regarding clinical outcomes, preliminary descriptive correlations suggested that patients with persistent *KRAS*/*NRAS* mutations were more likely to experience disease progression compared with those who achieved mutational clearance during therapy. Future large-scale studies integrating molecular and clinical data are warranted to verify and further explore these findings.

The present study provided valuable insights but also has several limitations. The relatively small cohort of 45 patients could constrain the generalizability of the findings, and serial plasma samples were not available at all-time points of disease progression. Subgroup analyses, such as comparisons between MSI-high and MSS tumors or between left- and right-sided CRC, could not be conducted due to limited sample size. Additionally, minor technical or pre-analytical variations could affect the results, and genomic alterations outside the targeted panel were not assessed. Despite these limitations, the results revealed a strong concordance between tissue- and plasma-based testing and successfully captured dynamic mutational changes during treatment. Larger, prospective studies integrating molecular dynamics with clinical outcomes are essential to validate the prognostic and therapeutic value of ct-DNA.

Looking ahead, the integration of ct-DNA analysis into routine clinical workflows holds considerable promise. ct-DNA testing could serve as a complementary approach to imaging and traditional tissue biopsies, particularly for monitoring minimal residual disease, anticipating relapse, and optimizing therapeutic sequencing, such as considering anti-EGFR rechallenge following clearance of *RAS* mutations. Beyond its clear clinical relevance, liquid biopsy also offers notable practical advantages, thus providing a minimally invasive, repeatable, and rapid alternative to tissue re-biopsy, eventually enhancing its suitability for real-world clinical practice.

A deeper understanding of ct-DNA biology can further advance its clinical application; however, the results of this study already underscored its significant value in metastatic CRC as a minimally invasive biomarker for capturing tumor heterogeneity, monitoring therapeutic resistance, and guiding personalized treatment strategies throughout the disease course (23,24).

In summary, the current study demonstrated that liquid biopsy using ct-DNA analysis represents a reliable and minimally invasive approach for the detection of tumor mutations in metastatic CRC. A high level of concordance was observed between plasma and tissue samples, particularly for *KRAS*, *NRAS*, and *BRAF*. In addition, dynamic changes in mutational status were detected during disease progression, thus highlighting the potential of ct-DNA for real-time monitoring of tumor evolution. These findings supported its role as a complementary tool to conventional tissue biopsy in guiding personalized treatment strategies. However, larger, prospective studies are warranted to validate these results and further establish the integration of liquid biopsy into routine clinical practice for patients with metastatic CRC.

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Availability of data and materials

The sequencing data generated in the present study may be found in the Zenodo repository at the following URL: https://doi.org/10.5281/zenodo.17742406. Raw instrument-level sequencing files (FASTQ or BAM) were not generated or retained during the Ion AmpliSeq workflow, as sequencing analysis and variant calling were performed directly within the Torrent Suite™ and Ion Reporter™ pipelines. Consequently,

only processed variant-level data (mutation calls, allele frequencies, and summary tables) are available and represent the full extent of the retained sequencing output. All analyses were performed for research and experimental purposes only and were not used for clinical decision-making; therefore, no formal clinical diagnostic reports were generated or archived. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

GZ was involved in the conceptualization and design of the study, data interpretation and drafting of the manuscript. MY participated in data collection, literature review and manuscript drafting. PN contributed to data collection, methodology, statistical analysis and manuscript editing. AT performed the laboratory analyses, validated the molecular testing results and contributed to data interpretation. IP performed the laboratory experiments, provided technical support and was involved in result validation. NT contributed to data collection, literature review and manuscript drafting. KD carried out statistical analyses, visualized data and critically reviewed the manuscript. GN contributed to the study design, interpretation of molecular and genetic data, and critically revised the manuscript for important intellectual content. EK contributed to the interpretation of clinical and genetic findings, provided input into study design, and critically revised the manuscript for important intellectual content. GP provided supervision, contributed to the conception of the study, and critically revised the final manuscript. GZ and MY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent for participation in this study. The study was conducted in accordance with the principles of The Declaration of Helsinki and was approved by the Institutional Review Board of the University Hospital of Ioannina (approval no. 11/18-04-2018).

Patient consent for publication

Written informed consent was obtained from the patients for the publication of this study and any accompanying images or clinical information. The patients reviewed the final version of the manuscript and agreed to its publication. Every effort has been made to ensure anonymity, and no identifying information is included in the publication.

Competing interests

GZ reports receiving personal fees as an invited speaker from Amgen, Ipsen, Leo Pharma and Merck. GP serves as the Chief Medical Officer for the European Society for Medical Oncology and is a member of the American Society of Clinical Oncology, the Hellenic Cooperative Oncology Group and the Hellenic Society of Medical Oncology. All other authors declare that they have no competing interests.

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