



Clonal diversity in *KRAS* mutant colorectal adenocarcinoma under treatment: Monitoring of cfDNA using reverse hybridization and DNA sequencing platforms

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ABSTRACT

Biological heterogeneity is a key feature of malignancies that significantly contributes to disease progression and therapy resistance. Residual/relapsed tumor foci may represent genetically divergent subclones, which remain uncovered as repeated and multiple tumor sampling is usually limited. The analysis of circulating free DNA (cfDNA) from the peripheral blood plasma (also called a liquid biopsy, LB) is a new achievement that provides an effective tool for follow-up monitoring of cancer-related genetic status. The present study highlights the phenomenon of mutational variability observed in patients with metastatic *KRAS* mutant colorectal cancer (mCRC) during treatment with bevacizumab in combination in a longitudinal fashion.

The prospective study included 490 mCRC patients evaluated between 2020 and 2022 in our institution. Out of the 211 *KRAS* mutant cases (43.06%) 12 tumors were identified with multiple *KRAS* gene variants (5.68%). Detailed follow-up investigations were possible in 3 of these patients including the genotyping of the primary and available metastatic tumors, and the peripheral blood cfDNA. cfDNA was collected from three different time points before and between cycles of combined treatment with bevacizumab chemotherapy. *KRAS* gene variants were identified using reverse-hybridization strips, and next-generation sequencing (NGS), and confirmed by conventional Sanger sequencing.

Interestingly, surgery and multiple treatment cycles reorganized the mutational profiles in the selected cases. The effect of the treatments resulted either in the overrepresentation of one of the pre-existing gene variants or in the appearance of new *KRAS* variants absent in the primary sample, according to the plasma cfDNA findings. Besides the *KRAS* variants demonstrated by targeted analysis, NGS mutational profiling identified some additional pathogenic variants from the cfDNA samples (including *NRAS* and *MET* alterations).

In conclusion, plasma cfDNA sampling enables the monitoring of mutational heterogeneity and subclonal dynamics of the actual metastatic tumor mass in mCRC. The pattern of molecular profile potentially reflects a differential drug response determining further progression.

1. Introduction

Circulating cell-free DNA (cfDNA) is a fraction of genomic DNA fragments released in the peripheral blood through active (e.g. cytotoxic cell lysis) or passive (e.g. necrosis) cell damage, also including cancer-derived DNA. According to the current view, cfDNA is a potential source of tumor DNA for genetic analysis, obtained by simple blood sampling which enables the real-time genetic assessment of the evolving malignant process [1–5]. Genetic progression is one of the leading causes of therapeutic resistance and treatment failure and has a major

effect on overall survival in cancer patients with metastatic disease [4, 6–8]. Genetically divergent subclones may evolve synchronously within the same tumor, or during disease progression (temporal heterogeneity) [4,5,9,10]. The selection of new subclones presenting with temporal heterogeneity may biologically influence the response to therapy and prognosis [5,11]. The DNA analysis of tumor tissue biopsies may reflect both intra-tumor and inter-tumor heterogeneity, the latter typically occurring in metastatic processes as a sign of progression. While tumor re-biopsy is increasingly critical during follow-up, the examination of plasma cfDNA has been reported as a simple alternative access to

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monitor cancer-related genetic alterations [4,5,9,12,13].

The clinical significance of cfDNA in patients with colorectal cancer (CRC) has already been investigated, and correlations with response to systemic therapy and treatment resistance were established [6]. According to the recent literature, cfDNA provides relevant prognostic and predictive information in different phases of CRC progression, including early diagnosis, residual disease detection, predictive biomarkers, and treatment response assessment [2,3,6]. It seems to be established, that mutation profiling using cfDNA reflects the dynamics of tumor evolution, and genetic heterogeneity in real-time and provides a genetic overview of the actual tumor mass also offering new therapy indications [14].

Regular cfDNA sampling enables to monitor the molecular response represented by the quantity of characteristic mutant alleles identified in the primary tumor sample. The follow-up can be done by monitoring one or several dominant gene variants, e.g. the specific *KRAS* variant as a major tumor biomarker in CRC. However, response to therapy, further to the loss or reappearance of individual gene variants, is complicated by the subclonal alternation of gene variants within the same oncogene, as it was already described also for the *KRAS* gene. According to the literature, multiple *KRAS* variants could be demonstrated synchronously in up to 5–10% of the *KRAS* mutant CRC cases [15]. The loss, reappearance, as well as alternation of different gene variants during cancer progression and treatment failure, may show highly individual patterns. Therefore, the comparative analysis of available primary and metastatic tumors and the peripheral blood cfDNA help together to understand the general features of subclonal progression and their interaction with the actual treatment applied.

Circulating-free DNA sampling and analysis became an everyday routine approach at our institution. With the accumulation of the data, we were increasingly faced with individual genetic patterns of CRC progression. Our prospective study aimed to (i) evaluate peripheral blood (PB) plasma cfDNA from patients with metastatic colorectal cancer (mCRC) with *KRAS* mutation, (ii) monitor the dynamics of *KRAS* variants during combined bevacizumab treatment, and (iii) demonstrate a relationship between the mutation status obtained from histological samples and paired cfDNAs originating in the same patients. For this purpose, all available primary and metastatic tumor samples, as well as cfDNA from three different time points (post-surgery/untreated, before cycle two and cycle four) were collected and subjected to quantitative mutational profiling by a reverse hybridization-based strip assay (Stripassay), Sanger sequencing and next-generation sequencing (NGS).

2. Materials and methods

2.1. Patients and study design

This was an observational study that included 490 patients with single or multiple metastases from colorectal adenocarcinoma undergoing primary sampling between September 2020 and August 2022 at the Department of Oncology, University of Debrecen. The 490 tissue samples were analyzed by *KRAS*, *NRAS*, and *BRAF* StripAssays. The histological samples (primer tumor and metastasis) of the three patients with multiple *KRAS* statuses were studied not only through Stripassays, but also Sanger-sequencing. NGS and StripAssays were performed on all liquid biopsy (LB) samples.

Altogether 211 *KRAS* mutant mCRC tumors were diagnosed (43.06%), out of which samples with multiple *KRAS* variants were selected (12/211; 5.68%). All criteria for patient follow-up were fulfilled in 3 cases where prospective peripheral blood sampling and cfDNA analysis was enabled. As a specific feature, these patients received combined bevacizumab chemotherapy due to the mutant *KRAS* status.

10 and 25 normal healthy individuals' cfDNA samples were analyzed and considered *KRAS* negative by the NGS panel and strip assay, respectively.

All protocols have been approved by the author's respective

Institutional Review Board for human subjects (IRB reference number: 60355-2/2016/EKU and 4648-6/2018/EÜIG).

2.2. Tumor DNA extraction from FFPE tissue samples

FFPE tumor tissue was selected for molecular analysis with a >10% tumor percentage determined in hematoxylin and eosin (H&E) stained slides by a pathologist. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The DNA concentration was measured in the Qubit dsDNA HS Assay Kit using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. cfDNA extraction from peripheral blood samples

Peripheral blood samples (liquid biopsy) were collected at three defined time points: pretreatment, before cycle two (range 14–21 days after starting therapy), and before cycle four (range 42–63). Briefly, blood samples were transferred in EDTA tubes (Becton Dickinson and Company, Sparks Glencoe, MD, USA) and processed within 2 h. The isolation process started with two centrifugation steps. First, blood tubes were centrifuged at 1300 g for 10 min. The collected supernatant was then centrifuged a second time to remove any possible remaining cells to eliminate debris at 16000 g for 10 min cfDNA was extracted from 5 mL of plasma into 40 µl of elution buffer using QIAamp® Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany). The DNA concentration was measured in the Qubit dsDNA HS Assay Kit using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The fragment size and distribution of cfDNA were measured using Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA, USA).

2.4. Sanger sequencing

For the Sanger-sequencing the exon 2 of the *KRAS* gene was amplified by PCR using the forward 5'-GGTACTGGTGGAGTATTGATAGTG-3' and reverse 5'-CGTCAAGGCACTCTTGCTAC-3' primers. The purified PCR products were sequenced by using the BigDye Terminator v1.1 Cycle Sequencing kit. The samples were analyzed on the ABI PRISM 310 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The variant allele frequency (VAF) of the Sanger sequencing electropherogram was calculated according to the following formula: mA% (proportion of mutant allele) = Hm (height of the mutant allele wave)/(Hm + Hwt (height of wild type allele wave))*100 [16].

2.5. StripAssay

Reverse hybridization was carried out using *KRAS* XL, *NRAS* XL, and *BRAF* 600/601 StripAssays according to the manufacturer's protocol (ViennaLab Diagnostics, Vienna, Austria). The assay covers 29 clinically relevant mutations in the *KRAS* gene, 22 mutations in the *NRAS* gene, and 9 alterations in the *BRAF* gene that are certified for human in vitro diagnostics (IVD). For interpretation, hybridization strips were aligned using the standardized layout supplied with the reagents, and positive bands were identified. The limit of detection of the reverse hybridization strip assays is 1% VAF.

2.6. Next-generation sequencing (NGS)

NGS libraries were created using the Archer® Reveal ctDNA™ 28 Kit (ArcherDX, Boulder, CO, USA). This panel is a targeted sequencing assay that simultaneously detects and characterizes single nucleotide variants (SNVs), insertions, and deletions (indels) in 28 genes associated with solid tumors. The panel includes the following 28 genes: *AKT1*, *ALK*, *AR*, *BRAF*, *CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ESR1*, *FGFR1*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MAP2K1*, *MAP2K2*, *MET*, *MTOR*, *NRAS*, *NTRK1*, *NTRK3*, *PDGFRA*, *PIK3CA*, *RET*, *ROS1*, *SMAD4*, and *TP53*. The final libraries were quantified KAPA library quantification kit (Roche, Basel,

Switzerland), diluted to a final concentration of 4 nM, and pooled by equal molarity. To sequence using the MiSeq System (MiSeq Reagent kit v3 600-cycle), all libraries were denatured by adding 0.2 nM NaOH and diluted to 40 pM with hybridization buffer from Illumina (San Diego, CA, USA). The final loading concentration was 10 pM libraries and 1% PhiX. Sequencing was conducted according to the MiSeq instruction manual. The data were analyzed with the Local Virtual Machine application of Archer DX Analysis software for the presence of SNVs and indels. The sequence quality for each sample was assessed. For the alignment, the human reference genome GRCh37 (equivalent UCSC version hg19) was built. The limit of detection of the assay was set at 1% variant allele frequency. A reliable variant detection requires minimum coverage of >250 reads. Individual gene variants were cross-checked in the COSMIC (Catalogue of Somatic Mutations in Cancer) and ClinVar databases for clinical pathogenicity.

3. Results

3.1. Multiple KRAS mutations in the local cohort and patient selection

Between October 2020 and September 2022 altogether 490 CRC samples were tested for KRAS exon 2, 3, and 4 gene variants, and 211 cases (43.06%) were identified with a previously described pathogenic gene variant. Within samples with mutation 12 cases (5.68%) presented with more than one KRAS variant in synchronous or metachronous fashion, all advanced cases with lymphatic or distant metastases of the liver and/or of the lung. Out of them, 3 cases were available for prospective sampling. From these patients, primary biopsy/surgical resection FFPE tumor samples, all available secondary tumors, and peripheral blood plasma from three different times were collected for molecular studies.

3.2. Mutational profiling of primary tumor and metastatic tissue samples

KRAS, NRAS, and BRAF mutational status of primary tumor samples was tested using high-sensitivity reverse hybridization technology (StripAssay) in all available tissue samples from Case 1–3. Mutations identified by this primary testing were confirmed by Sanger sequencing. The molecular genetic results are presented in Table 1.

The VAF of Sanger electropherograms was determined in all histological samples with >5% VAF as the lower limit of sensitivity. The range of the VAF of KRAS mutations was in the range between 5 and 50% (mean 19.3%).

3.3. cfDNA concentrations and mutational profiling of liquid biopsy samples

cfDNA was isolated from 5.0 ± 0.5 mL PB plasma samples before the start of the combined chemotherapy as well as before cycles two and four of the therapy regimen. The cfDNA yields ranged from 0.6 to 55.0 ng/ μ L (mean 6.33 ng/ μ L), indicating the high variability in DNA content. However, DNA quantities did not differ from our routine approach and were appropriate to represent tumor-specific changes.

cfDNA from cases 1–3 presenting with multiple KRAS variants was tested in all plasma samples using reverse hybridization strips first. NGS was performed then to validate cfDNA mutational profiles and allele frequencies (Table 1.).

4. Case presentations

4.1. Case 1

A 66-year-old man with abdominal symptoms, bleeding, typical right colon tumor, multiple liver foci, loco-regional, mesenteric, and hepatic portal lymph node metastases described by CT findings was admitted to the surgery department. A right hemicolectomy was performed and a Grade 2 adenocarcinoma with a pathological stage pT3, pN1a with 1/15 positive lymph nodes was diagnosed. The patient was started on bevacizumab and xelox therapy because of the positive KRAS mutation status. The first liquid biopsy (LB) plasma sample was collected 33 days after surgery.

KRAS mutation profile of the primary tumor resection samples (R1–R5), metastasis (M), and the three PB cfDNA were analyzed (Table 1., Fig. 1.). In R3 and R5 samples two KRAS variants were detected (c.34G > T; p.Gly12Cys and c.35G > T; p.Gly12Val). In sample R1 only the c.34G > T; p.Gly12Cys mutation was identified, while in samples R2, R4, and M the other genotype could be found (c.35G > T; p.Gly12Val). In the first and second LB plasma samples (LB1, LB2) KRAS c.34G > T; p. Gly12Cys aberration was identified, while the third plasma cfDNA (LB3) remained negative for KRAS alteration.

Our results obtained with StripAssay from liquid biopsy were further validated by NGS. The 28 genes ctDNA panel analysis identified KRAS mutation c.34G > T; p.Gly12Cys as the single pathogenic variant in samples LB1 and LB2 with frequencies VAF 3.0% and 1.5%, in complete agreement with the reverse hybridization screening method. The last plasma sample (LB3), similar to the result of the reverse hybridization assay, demonstrated none of the previously described mutations.

Table 1

Results of KRAS genotyping from tissue and liquid biopsy (LB) samples in three CRC cases presenting with multiple KRAS variants.

Patients	Sample	Sample type ^a	KRAS genotype (Sanger and StripAssay)	Sanger VAF%	Tumor cell ratio (%)	KRAS StripAssay genotype (VAF%) ^b		
						LB1	LB2	LB3
1	R1–R5	colon	c.35G > T; p.Gly12Val	25	30	c.34G > T; p. Gly12Cys (3)	c.34G > T; p. Gly12Cys (1.5)	NMD
			c.34G > T; p.Gly12Cys	10	30			
	M	lymph node metastasis	c.35G > T; p.Gly12Val	5	30			
2 ^c	TB	rectum	c.38G > A; p.Gly13Asp	5	10	c.35G > T; p. Gly12Val (2) c.35G > A; Gly12Asp (2.5)	c.35G > C; p. Gly12Ala (1.5)	c.35G > C; p. Gly12Ala (2) c.35G > A; Gly12Asp (2.5)
3	R	colon	c.38G > A; p.Gly13Asp	30	30	c.38G > A; p. Gly13Asp (2.5)	c.34G > T; p. Gly12Cys (2) c.35G > A; p. Gly12Asp (2)	c.34G > T; p. Gly12Cys (1.5)
	M1	lymph node metastasis	c.34G > T; p.Gly12Cys	25	30			
	M2	pancreas	c.38G > A; p.Gly13Asp	15	20			

^a Primer tumor and available metastasis samples, in all patients metastasis was detected using CT image analysis (R: resection, M: metastasis, TB: tissue biopsy).

^b Variant allele frequency (%) determined by NGS.

^c Besides KRAS alteration NRAS mutation was detected in the primary tumor biopsy sample (additional MET and NTRK1 mutations were identified in the plasma cfDNA, see Table 2).

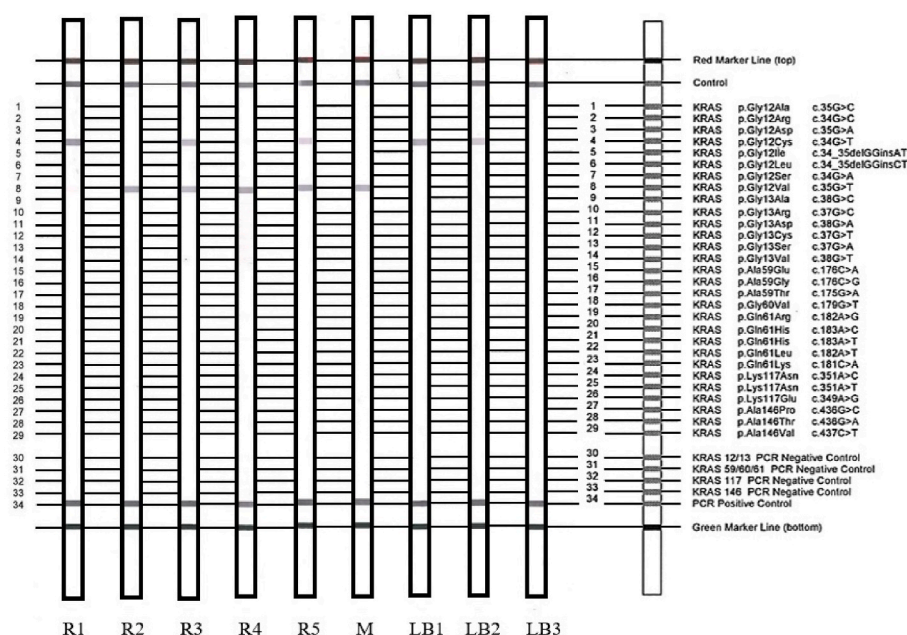


Fig. 1. Results of *KRAS* reverse hybridization Strip-assay (Case 1) reflected by the evaluation template enabling the exact determination of gene variants. Two types of codon 12 *KRAS* variants were identified at the time of the primary surgery (R1-R5 representing tissue samples from the resection material, M representing a metastasis from a lymph node). Variant c.34G > T; p.Gly12Cys was detected in plasma cfDNA before treatment (LB1) and before cycle two (LB2). Before cycle four the mutant genotype disappeared from the plasma (LB3). R: resection material, M: metastasis, LB: liquid biopsy.

4.2. Case 2

A 70-year-old man was examined by rectoscope due to a tumor mass next to the anus causing nearly complete stenosis. Whole-body imaging

(CT and MRI) revealed tumor mass in the rectum area with regional perirectal involvement, lymphadenomegaly and multiplex hepatic metastasis. Pathological examination of the biopsy identified adenocarcinoma Grade G2, based on which diagnosis first-line neoadjuvant

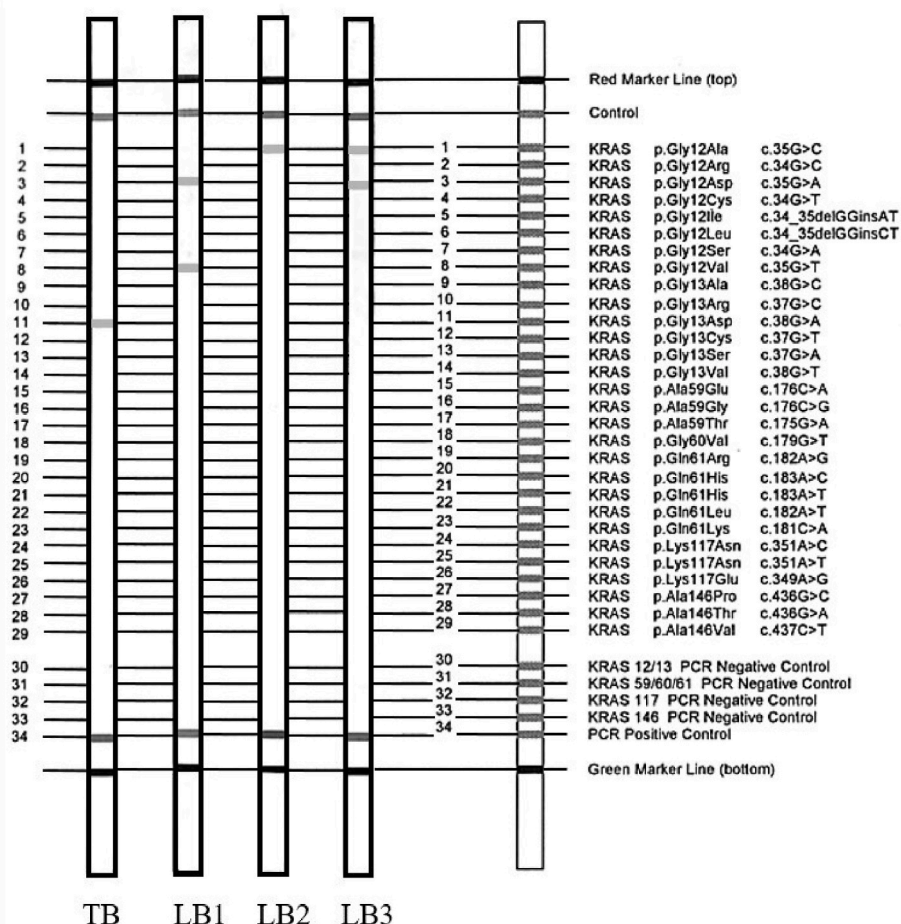


Fig. 2. Results of Case 2 *KRAS* reverse hybridization Strip-assay. TB: tissue biopsy; LB: liquid biopsy.

Table 2

NGS-based mutation profile of the primary tissue biopsy (TB) and the three post-treatment plasma cfDNA samples (LB1-3) from Case 2.

Affected gene	Nucleotide change	Amino acid change	Origin of DNA (VAF %)				Clinical significance ^a
			TB	LB1	LB2	LB3	
<i>KRAS</i>	c.38G > A	p.Gly13Asp	6.7	0	0	0	pathogenic
	c.35G > T	p.Gly12Val	0	2.0	0	0	pathogenic
	c.35G > A	p.Gly12Asp	0	2.5	0	2.5	pathogenic
	c.35G > C	p.Gly12Ala	0	0	1.5	2.0	pathogenic
<i>MET</i>	c.2908C > T	p.Arg970Cys	48.0	45.0	49.0	49.0	likely benign
<i>NRAS</i>	c.37G > C	p.Gly13Arg	24.3	0	0	0	pathogenic
<i>NTRK1</i>	c.1702C > T	p.His568Ter	53.0	46.0	52.0	54.0	uncertain significance
	c.1730G > T	p.Gly577Val	49.0	47.0	54.0	42.0	uncertain significance

^a The pathogenicity was determined using COSMIC or Clinvar databases.

radiochemotherapy was given. Lymph node and liver metastases were not resectable, so no surgery has been performed to date. Bevacizumab with xelox treatment was started 189 days after the initial diagnosis, the first blood sample (LB1) was taken at this time.

Reverse hybridization and NGS-based sequencing of the primary tumor detected a *KRAS* c.38G > A, p.Gly13Asp and an *NRAS* c.37G > C, p.Gly13Arg variant. In contrast, the first plasma sample, following the first line neoadjuvant treatment (LB1) presented with two new *KRAS* mutations at c.35G > A; p.Gly12Asp and c.35G > T; p.Gly12Val. Interestingly, in the second cfDNA (LB2) only variant c.35G > C; p.Gly12Ala appeared. In the third plasma sample (LB3) beside c.35G > C; p.Gly12Ala variant c.35G > A; p.Gly12Asp reappeared just like in sample LB1. However, no *NRAS* mutations were found in any of the plasma cfDNAs (Fig. 2).

Besides the RAS variants, the following gene mutations could be detected using NGS: *MET* c.2908C > T; p.Arg970Cys, *NTRK1* c.1702C > T; p.His568Ter and *NTRK1* c.1730G > T; p.Gly577Val, which were continuously present throughout the follow-up samples. Near 50% of VAF suspected a germline origin of these alterations, therefore, a tumor-

free liver biopsy sample was tested to confirm the germline origin.

The *MET* NM_000245.4:c.2908C > T (p.Arg970Cys) variant is also known as NM_001127500.3:c.2962C > T (p.Arg988Cys) and is listed 23 times in ClinVar, mostly enrolled as likely benign. The *NTRK1* NM_002529.4:c.1702C > T variant is annotated as p.Gln568Ter with uncertain significance. The *NTRK1* NM_002529.4:c.1730G > T (p.Gly577Val) variant is identified as c.1712G > T using NM_001012331.2. The molecular profile and VAF values obtained from the four-time points (TB and LB1-3) tested are displayed in [Table 2](#).

4.3. Case 3

A 77-year-old man was submitted to colonoscopic examination in 2015 resulting in an occluding tumor of the transverse colon with a biopsy finding of adenocarcinoma. Left hemicolectomy with a metastatic lymph node was performed resulting in adenocarcinoma grade G3, pT3, pN2b, and mutant KRAS status. After surgery, the patient received first-line adjuvant chemotherapy (Folfox), distant metastases and residual tumor were not demonstrated at that time. In 2020, four years and

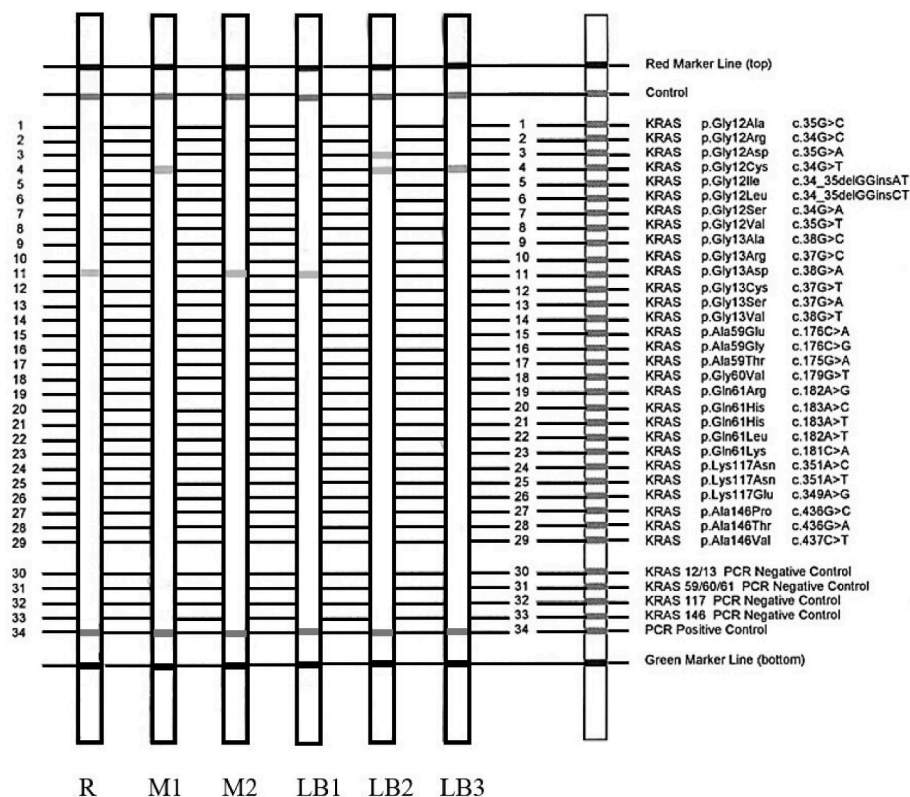


Fig. 3. Case 3 StripAssay template representing the appearance of *KRAS* variants in tumor resection sample (R), in lymph node metastasis from the time of the initial surgery (M1), in metastatic pancreas tumor biopsied 53 months later (M2), and plasma cfDNA (LB1-3).

five months later an abdominal CT examination uncovered a solid lesion in the pancreas, which was subsequently sampled. The histology and immunophenotype confirmed the metastatic nature originating from the colon adenocarcinoma. Bevacizumab and xelox therapy were decided before which the first peripheral blood sample (LB1) was collected. Similar to the previous cases cfDNA testing was repeated after cycles two (LB2) and four (LB3) of the therapy regimen.

The primary tumor and pancreas metastasis presented with the identical c.38G > A; p.Gly13Asp *KRAS* variant while the lymph node metastasis from the time of the primary surgery carried c.34G > T; p. Gly12Cys variant. The original c.38G > A; p.Gly13Asp aberration was identified only in the first PB plasma cfDNA (LB1). In the second plasma sample (LB2) two new variants, c.34G > T; p.Gly12Cys (VAF: 2%) and c.35G > A; p.Gly12Asp (VAF: 2%) were identified. Moreover, in the third cfDNA, only the c.34G > T; p.Gly12Cys (VAF: 1.5%) aberration has appeared (Fig. 3).

5. Discussion

The development and spatial redistribution of tumor subclones is a continuous process in progressive neoplasias and different compartments may respond differentially to anti-tumor therapies. Moreover, chemo-radiotherapy potentially triggers the selection of resistant clones and induces the generation of new mutations and other gene rearrangements contributing to increased survival. Consequently, the analysis of a single tumor sample is unlikely predictive of what happens later in secondary lesions and generally reflects only a spatiotemporal snapshot of the whole tumor [4,13]. From this perspective, the genetic monitoring of residual tumors and newly formed metastases is of high clinical impact. While repeated tumor sampling upon progression is strongly recommended, it is complicated by numerous factors. The recently available approach of liquid biopsy enables the analysis of plasma cfDNA released from the malignant proliferate. One of the major advantages is continuous access to genetic material. As a special feature, cfDNA genotyping reflects a composite genetic picture representing genetic subclones, independent of their actual anatomical localization. It is not surprising, that the monitoring of the actual mutational status uncovers the diversity and the sometimes unexpected variability of subclones highlighted by the gene variants tested.

In our study, we were interested in the real-life dynamics of multiple *KRAS* variants identified in colorectal carcinoma samples. Although the number of patients available for detailed profiling was strongly limited, highly interesting observations were made. Variant diversity was uncovered in 5.68% of our *KRAS*-mutant mCRC cohort, also indicating that the subclonal variation of the mutational profile is a real-life feature of mCRC. Patient 1 showed intratumor heterogeneity with subclonal evolution in the primary tumor examined. The five separate primary tumor samples (R1-R5) variably reflected two pathogenic *KRAS* variants, with only two samples featuring both of them (R3 and R5). Interestingly, the single surgically removed lymph node metastasis presented with the single variant c.35G > T; p.Gly12Val, which was different from the only variant detected in plasma cfDNA (c.34G > T; p.Gly12Cys) before and after initiation of the combined therapy. After the radical removal of the primary tumor and adjacent lymphatic metastases, the liquid biopsies should only represent pathogenic variants of the residual tumor mass. The *KRAS* variant detected in LB1 and LB2 was provided as a single change in two (R1 and R3) of the primary tumor areas. This suggests that residual tumor foci overcoming surgery derive from R1/R3 or biologically similar areas. The third cfDNA sample (LB3) remained free of any variants, which can be attributed to treatment response, while temporary sampling failure, also reported in association with LB, can also not be excluded. Raimondi et al. examined eleven RAS mutant mCRC cases with only 6 (55%) presenting the same variants in the plasma, while the rest was found wild-type (5 cases, 45%). It is of clinical importance that patients testing negative for RAS mutations in plasma at the time of disease progression were considered eligible for treatment with EGFR

inhibitors [17]. In Patient 2 the only tumor sample provided for testing presented with a single *KRAS* and further variants of the *NRAS*, *MET*, and *NTRK1* genes. However, LBs taken after the neoadjuvant radio-chemotherapy and during bevacizumab combination therapy reflected three additional *KRAS* and no *NRAS* variants, referring to large-scale tumoral heterogeneity upon anti-tumor therapy. *MET* and *NTRK1* mutations were identified through all cfDNA samples with the same high-frequency VAF indicating a germline origin. As an important feature of the case, the primary rectal carcinoma was irresectable and the patient gained multiple hepatic metastases. To our hypothesis, the founder clone developed in subclones with diverse *KRAS* variants. The single small biopsy sample from the primary most likely served as a limited resource bearing only one of the *KRAS* variants. The relatively low allele frequency (6.7%) determined here from the tumor DNA could be interpreted as a subclonal change, as suggested by the statistical distribution of gene variants in our database. Different areas of the same primary and metastatic tumors might have progressed independently by gaining other pathogenic variants, potentially also triggered by the applied genotoxic therapy. The dynamic switch of the gene variants is still surprising, but not an isolated finding. As an example, Sclafani F. et al. compared *KRAS* status between tumor tissue and plasma samples in patients with locally advanced rectal cancer and came to discrepant plasma results in 26.8% of their cases, all verified later by extended tissue sampling [18].

In Patient 3 the surgically resected primary tumor and lymph node metastasis, as well as late distant organ metastasis (pancreas) could be compared, followed by LB cfDNA testing. While the primary tumor (R), the pancreas metastasis (M2), and cfDNA from the plasma before bevacizumab (LB1) showed the same *KRAS* mutation, the lymph node metastasis from the time of the initial diagnosis (M1) and follow-up cfDNAs (LB2 and LB3) from later time points shared the other *KRAS* variant, with an additional, third *KRAS* variant appearing at a single occasion in the cfDNA sample LB2. A switch from one to the other variant is acceptable in a recurrent metastatic tumor after therapy, especially with matching results from the plasma cfDNA, as seen also in the previous example. However, consecutive plasma results returning to the original variant (with some variation) is a finding that is confusing and can only be explained by the extreme temporospatial complexity of subclones existing side-by-side.

The three cases represent different situations where new *KRAS* variants come into sight and/or disappear during the disease. In Case 1 multiple samples from the primary tumor were sequenced allowing a deeper insight into the subclonal setting. In Cases 2 and 3 only one tumor sample was provided before therapy featuring a single *KRAS* variant, which was followed by the detection of extra variants during the disease. Within these three mCRC cases, 2, 3, or 4 known *KRAS* variants were detected in the different samples. The genetic profile might be strongly influenced by tumor heterogeneity, aggressiveness, and therapy response. In addition, variant sets detected in the plasma may be the result of response/resistance to therapy on a clonal/subclonal basis. However, a positive result indicates the existence of the particular variant and thus, the multiplicity of the subclones.

While reports generally favor the choice of cfDNA analysis for diagnostic and monitoring purposes in mCRC [19], much fewer data are provided on the issue of genetic variability. As such, Furiki et al. examined emerging mutated genes in mCRC by collecting primary tumors, metastatic liver tumors, and liquid biopsies from 22 patients [9]. They concluded, that liver metastases partially present with emerging mutations that were not present in the primary tumors but were likely to be detected in ctDNA using liquid biopsy. All mutations detected using liquid biopsy were identified in metastatic liver tumors, even if they were not detected in the primary tumors. While the concordance rate between primary and metastatic tumors is claimed to be generally high, the estimated probability of emerging mutations in metastatic tumors is estimated at around 10% [9,20,21].

The direct comparison of cfDNA versus tumor DNA repeatedly

illustrated, that single biopsies frequently fail to identify the presence of multiple clinically relevant predictive features. Detailed sequencing of cfDNA suggested additional concurrent resistance mechanisms in 78% of cases [22]. cfDNA regularly reflects clinically relevant alterations in a heterogenous fashion, becoming critical to the subsequent therapy planning. As a classical example, monitoring the onset of secondary resistance to *anti*-EGFR therapy can be effectively based on LB [22–24]. Similarly, an LB-based approach to the *KRAS* mutant status following surgery and first-line treatment may argue for the use of an *anti*-EGFR therapy [25]. Moreover, biological therapies directed to specific pathogenic *KRAS* variants are expected with the requirement of accurate follow-up of the *KRAS* status in the near future.

In summary, LB is to be considered a great supplement in addition to tissue biopsy, which remains a gold standard for the diagnosis and predictive testing of solid tumors. LB provides a different kind of information including a general insight into the dynamics of the genetically heterogeneous malignant process. With the cfDNA released to the peripheral blood, access to all compartments of the tumor is enabled irrespective of their spatial and temporal distribution. Although many aspects of tumor-derived cfDNA are not clear yet, the technology is getting widely available in parallel with increasing clinical attendance. The presented examples illustrate the beginning of an area where genetic and functional features of solid tumors - like mCRC - should be regularly monitored for response failure and treatment optimization.

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Institutional Review Board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of the University of Debrecen (protocol code 60355-2/2016/EKU and 4648-6/2018/EÜIG).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

CRedit authorship contribution statement

Emese Sarolta Bádon: and. **Attila Mokánszki:** Conceptualization, Methodology, Visualization, Writing – original draft. **Anikó Mónus:** Methodology. **Csilla András:** Data curation, sample contribution. **Gábor Méhes:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Supervision, Writing – review & editing, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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