Molecular and Cellular Probes 67 (2023) 101888

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Identification of liquid biopsy-based mutations in colorectal cancer by targeted sequencing assays

István Szász [a,b], Tímea Kiss [a,b], Attila Mokánszki [c], Viktória Koroknai [a,b], János Deák [d], Vikas Patel [e], Krisztina Jámbor [b,e], Róza Ádány [a,b], Margit Balázs [a,b,*]

[a] ELKH-DE Public Health Research Group, University of Debrecen, 4032, Debrecen, Hungary
[b] Department of Public Health and Epidemiology, Faculty of Medicine, University of Debrecen, 4032, Debrecen, Hungary
[c] Department of Pathology, Faculty of Medicine, University of Debrecen, 4032, Debrecen, Hungary
[d] University of Debrecen Clinical Center, Surgery Clinic, University of Debrecen, Debrecen, Hungary
[e] Doctoral School of Health Sciences, University of Debrecen, Hungary

ARTICLE INFO

Keywords:
Colorectal cancer
Tumor tissue and plasma samples
cfDNA
Next generation sequencing
Liquid biopsy

ABSTRACT

Recently, liquid biopsy, as a promising approach was introduced for the analysis of different tumor-derived circulating markers including tumor DNA and cell free DNA (ct/cfDNA). Identification of mutations in cfDNA may allow the early detection of tumors, as well as predicting and monitoring treatment responses in a minimally invasive way. In the present study, we used commercially available gene panels to verify the mutation overlap between liquid biopsy and abnormalities detected in colorectal tumor tissue. The two panels (Archer®VariantPlex®Solid Tumor and LIQUIDPlexTM ctDNA) overlap in 23 genes, which enables a comprehensive view of tumor-plasma mutational status by next generation sequencing. We successfully analyzed 16 plasma and 16 tumor samples. We found that 87% of tumor tissues contained 44 mutations in 12 genes and 43.8% of cfDNA harbored 13 mutations in 5 genes. To verify whether the mutation pattern of the tumor DNA could be consistently detected in plasma cfDNA, we compared the alterations between cfDNA and matched tissue DNA in nine patients. Six of the 9 tumor tissues harbored mutations in *TP53, KRAS* or *MET* genes, those were not detectable by the ctDNA kit, even eventhough the exons of these genes overlap in both panels. Comparing the mutational patterns of the matched samples, we found that only one cfDNA had the same mutations (*KRAS, SMAD4* and *TP53*) in the paired tissue. The results of the comparison between tumor tissue DNA and matched plasma cfDNA underline the importance of studying the paired solid tumor and plasma samples together.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, according to the World Health Organization GLOBOCAN database. Rates of both incidence and mortality are substantially higher in males than in females [1]. The prevalence of colorectal cancer is influenced by many different factors and varies from country to country [1]. Hereditary colorectal cancer syndromes, such as familial adenomatous polyposis (FAP), MUTYH-associated polyposis (MAP) and Lynch syndrome are responsible for only five percent of CRC patients [2]. A significant proportion of colorectal tumors are caused by well-known risk factors such as inflammatory bowel disease, obesity, sedentary lifestyle, tobacco and alcohol use, immunosuppressive medications, diabetes, etc. Although colorectal cancer incidence has decreased in the overall population over the last fifty years, it has increased by two percent in those under-fifty, which suggests a significant increase in projected incidence of early-onset CRC by 2030 [3,4].

Although liquid biopsy was originally used in connection with the analysis of circulating tumor cells (CTCs), nowadays it has been extended and referred to the analysis of also circulating cell-free DNA (cfDNA) or RNA, and exosomes from blood, urine, pleural effusion, ascites, or cerebrospinal fluid [5,6]. Examination of these body fluids gives a real-time picture of tumor-associated changes in patients. These

* Corresponding author. ELKH-DE Public Health Research Group, University of Debrecen, 4032, Debrecen, Hungary.
E-mail addresses: szasz.istvan@med.unideb.hu (I. Szász), kiss.timea@med.unideb.hu (T. Kiss), mokanszki.attila@med.unideb.hu (A. Mokánszki), koroknai.viktoria@med.unideb.hu (V. Koroknai), deak.janos@med.unideb.hu (J. Deák), vikas.patel@med.unideb.hu (V. Patel), jambor.krisztina@med.unideb.hu (K. Jámbor), adany.roza@med.unideb.hu (R. Ádány), balazs.margit@med.unideb.hu (M. Balázs).

https://doi.org/10.1016/j.mcp.2022.101888
Received 15 November 2022; Received in revised form 6 December 2022; Accepted 6 December 2022
Available online 10 December 2022
0890-8508/© 2023 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

samples can be used to estimate the possibility of metastasis formation or tumor progression, sensitivity to anticancer agents, monitoring of treatment response, and identification of minimal residual disease (MRD) [7]. In addition, liquid biopsy also provides useful information about drug resistance mechanisms [8–10]. Its use has many advantages, the most important being that it is a minimally invasive, therefore multiple sampling allows continuous monitoring of the disease. The other crucial advantage over tissue biopsy is that it makes easier to study the heterogeneity of the tumor, which is often underestimated [11]. The heterogeneity of the tumor is the main reason for the development of resistance to anticancer agents and resulting treatment failure. On one hand, the heterogeneity can be spatial when genetic diversity occurs in the cells within the tumor or between primary-metastatic or metastatic-metastatic tumors within the same patient. On the other hand, it can be temporal when genetic changes in tumor cells are mainly triggered by anticancer drugs [12–14]. In our study, we isolated cfDNA from colorectal patients' blood samples. These DNA fragments mainly enter the bloodstream through apoptosis and necrosis of tumor cells, while normal cfDNA fragments come predominantly from presumably normal white blood cells of the host [15]. Specific isolation of normal and tumor-derived cfDNA is not possible, but cancer patients have higher concentrations of cell-free DNA. Furthermore, cfDNA fragments smaller than 100 bp are more frequently present in CRC patients, whereas the larger fragments (>300 bp) are more typical of healthy individuals [16,17].

Our experiments involved the sequencing of 16 colorectal tumor samples using Archer® VariantPlex® Solid Tumor Kit which simultaneously detects and characterizes single nucleotide variants (SNVs), copy number variations (CNVs) and insertions/deletions (indels) in 67 genes associated with solid tumors. For the sequencing of 16 plasma samples (9 in pairs with colorectal tumor samples) we used the LIQUIDPlexTM ctDNA 28 Kit which is an advanced solution for targeted next-generation sequencing (NGS) of circulating cell-free tumor DNA (ccfDNA/cfDNA/ctDNA) containing 28 genes commonly associated with solid tumor type cancers. The two panels have an overlap in 23 genes which enables a more comprehensive view of tumor tissue - plasma originated cfDNA mutational status.

The aim of our study was to compare the genetic alteration patterns of colorectal tumor tissues to alterations present in colorectal tumor patients peripheral blood. The main advantage of this recently implemented liquid biopsy assay is that this is a minimally invasive sample collection method. On the other hand, we aimed to compare the mutation patterns of tumor DNA and plasma cfDNA of the same patient. If the results obtained with the two different techniques shows a high degree of similarity, it would provide an opportunity to use the liquid biopsy for diagnosis and therapy selection. However, if the results are divergent the combined use of the samples may be most beneficial for patients.

2. Materials and methods

2.1. Plasma separation for cfDNA isolation

Plasma separation was performed within 2 h after blood collection in all cases. The blood was collected into BD Vacutainer® Venous Blood Collection Tubes (cat. no. 367525) containing EDTA and then poured into 50 mL falcon tubes and was centrifuged at 4 °C, 3000 rpm, 10 min. The supernatant was then carefully poured into Eppendorf tubes and centrifuged at 4 °C, 16000 g, 10 min. The 5 mL aliquots were stored at −80 °C until use.

2.2. cfDNA isolation

For cfDNA isolation QIAamp ccfDNA/RNA Kit (QIAGEN, Hilden, Germany) was used. The isolation was carried out according to the manufacturer's instructions. Plasma samples were transferred (5 mL) into a 15 mL collection tube, and 300 μL Buffer RPL (buffer for lysis of proteins, exosomes and inactivation of RNases) for every 1 mL of plasma was added for 3 min. Then 100 μl Buffer RPP (buffer for precipitation of proteins and other contaminants) was added to every 1 mL of plasma and vortexed for at least 20 s and incubated on ice for 3 min before the samples were centrifuged at 3000×*g* for 10 min to pellet the precipitate. The supernatants were transferred into a new tube and 1 vol ice-cold isopropanol was added to each and mixed. Samples were pipetted onto RNeasy Midi spin columns and centrifuged at 3000×*g* for 3 min at room temperature, the flow-through was discarded. Four mL Buffer RWT (a stringent washing buffer with ethanol used after preclearing the sample) were added to the RNeasy Midi spin columns and centrifuged for 3 min at 3000×*g* and the flow-through were discarded. Two point five mL of RPE Buffer (a mild washing buffer, and a proprietary component of RNeasy Kits) was pipetted onto each RNeasy Midi spin column and centrifuged for 15 min at 3000×*g*, the flow-through was discarded. 200 μL RNase-free water was directly added to the center of the spin column membrane and incubated for 1 h, then centrifuged at full speed for 1 min to elute the cfDNA.

For cfDNA cleanup 200 μL RPL buffer and 800 μl ethanol (96%) was added to the elution and mixed. This mixture was transferred on RNeasy MinElute spin column and was centrifuged for 12000×*g* 1 min. For the membrane washing 500 μL Buffer RPE was added onto the RNeasy MinElute spin column and centrifuged for 12000×*g* for 1 min the flow-through was discarded. This step was repeated with 500 μl of 80% ethanol. Finally, the membrane was dried using the opened spin column for 5 min. RNase-free water (20 μL) was used to elute the DNA and after 1 min incubation, centrifuged for the columns 1 min at full speed.

2.3. DNA isolation from frozen CRC samples

For DNA isolation from the CRC tumor samples NucleoSpin Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) was used. The isolation was carried out according to the manufacturer's protocol. Tumor samples were homogenized using MagNA Lyser for 40 s at 6000 rpm. Samples were pre-lysed adding 180 μL T1 buffer and 25 μL proteinase K, and incubated at 56 °C overnight. First 200 μL buffer B3 was added and incubated the samples at 70 °C for 10 min. In the next step 210 μL 96% ethanol was added, and placed on NucleoSpin® Tissue column and centrifuged for 1 min at 11000×*g*. The membranes were washed first with 500 μL BW buffer, after with 600 μL B5 buffer. Finally, the membrane was dried (each step at 1 min at 11000 x rpm). The DNA was eluted with 100 μL prewarmed RNase-free water (1 min at 11000 x rpm).

2.4. Next-generation sequencing (NGS) and data analysis

Next-Generation Sequencing was performed as described by Mokánszki et al. [18]. Archer PreSeq DNA Calculator (Archer DX, Boulder, CO, USA) was used to determine the amount of amplifiable DNA. For library preparation, Archer VariantPlex Solid Tumor Kit covering 67 genes, and Archer LIQUIDPlexTM ctDNA 28 Kit (Archer DX, Boulder, CO, USA) were performed. The final quantification of the libraries was carried out using KAPA Universal Library Quantification Kit (Kapa Biosystems, Roche, Basel, Switzerland). The MiSeq System (MiSeq Reagent kit v3 600 cycles, Illumina, San Diego, CA, USA) was used for sequencing. After denaturation, the final loading concentration was 8 pM libraries and 1% PhiX. Captured libraries were sequenced in a multiplexed fashion with a paired-end run. The sequencing read cutoff was determined at least 250X depth of coverage. The fastq files were analyzed with Archer analysis software (version 6.2.; Archer DX, Boulder, CO, USA) for the presence of single-nucleotide variants (SNVs) as well as insertions and deletions. ("indels"). The human reference genome GRCh38. p14 was built for the alignment. Variant allele frequency cutoff was set to 5%. Large insertion/deletion (>50 bp) and chromosomal aberrations could not be captured by the method. Nucleotide and amino acid changes were described using the latest version of

Human Genome Variation Society nomenclature.

List of gGenes tested on the Archer VariantPlex Solid Tumor Kit a targeted sequencing assay to that simultaneously detects and characterizes single nucleotide variants (SNVs), copy number variations (CNVs), and insertions and deletions (indels) in 67 genes associated with solid tumors are summarized listed in Table 1 A, and list of genes on the included into the Archer LIQUIDPlexTM ctDNA panel are summarized presented in Table 1 B.

3. Results

3.1. Patient characteristics

Twenty-three patients diagnosed with primary or metastatic CRC were included in the study. Histopathological characteristics of the patients and tumor samples are summarized in Table 2. Patients included 13 males (56%) and 10 females (44%), with a median age of 67 years (range 50–86). The tumors differed in histological and biological properties and had a tumor size between 2.2 and 16 cm. Seventy-nine percent (n = 18) of the patients had primary tumors, half of these with ulcerated surfaces and 21% (n = 5) had liver metastases at the time of blood sampling.

All tumor samples were handled according to the rules and regulations by the University of Debrecen, Hungary with the approval of the Hungarian Ethics Committee (TUKEB 17876–2018/EKU). Histological examination and tumor material selection were done by trained pathologists in the Department of Pathology. Tumor samples with the highest quality (i.e. the representation of tumor cells was >80%) were included in the study. Tumor DNA and plasma cfDNA were available from the same patient in nine-; only plasma cfDNA from six- and only tumor DNA from seven patients. Despite high tumor cell representation in the tumor tissue in two patients (P46 and P64), cfDNA was not detectable in plasma sample.

3.2. Genetic analysis of solid tumor samples

Sixteen colorectal tumor samples were sequenced using the Archer® VariantPlex® Solid Tumor 67 gene panel. Fourteen CRC tissues (87%) harbored 44 mutations in 12 genes (1–6 mutations/sample; with a median mutation of 3). In two samples (samples P45 and P51) we could not find any mutations in the 67 genes examined. Majority of the tumors (n = 13), exhibited mutations in three genes: *TP53* (n = 10), *APC* (n = 10) and *KRAS* (n = 6). Thirty-two (73%) of the mutations were missense, associated with an amino acid exchange; eight (18%) were nonsense mutation; three (7%) were frameshift mutation and one (2%) was in-frame deletion (Supplementary Table 1). The variant allele frequency and the distribution of the mutations were highly different between samples. The highest variant allele frequency (VAF) was observed in the *TP53* gene variant (0.749) in tumor sample P49. Fig. 1 summarizes the number of gene mutations and variants identified by next generation sequencing. Detailed description of all types of mutation with genomic location and clinical significance in tumor tissue are summarized in Supplementary Table 1.

The relationship between the number of gene alterations and clinical parameters of patients are summarized in Table 3. The *TP53* tumor suppressor gene was altered in 10 patients and the number of mutations were 13. The other frequently mutated genes were the *APC* in 8 patients (number of mutations within the gene was 8) and *KRAS* in 6 patient's tumor tissues. Examining the relationship between clinical parameters and gene mutations in the samples, we observed that mutations of two genes (*NOTCH1* and *FOXL2*) were associated with T3 stage (Table 3).

Table 1
Gene targets on the Archer VariantPlex Solid Tumor and LIQUIDPlexTM ctDNA 28 Kit. * genes that are common on both panel are highlighted with bold in grey cells

A. Genes on the Archer VariantPlex Solid Tumor panel						
ABL1	*CDK4*	*EZH2*	*H3F3A*	***MAP2K1***	***PDGFRA***	*SMARCB1*
AKT1*	*CDKN2A*	*FBXW7*	*HNF1A*	*MDM2*	***PIK3CA***	*SMO*
ALK	*CSF1R*	***FGFR1***	***HRAS***	***MET***	*PIK3R1*	*SRC*
APC	***CTNNB1***	*FGFR2*	***IDH1***	*MLH1*	*PTEN*	*STK11*
ATM	***DDR2***	*FGFR3*	***IDH2***	*MPL*	*PTPN11*	*TERT*
AURKA	***EGFR***	*FLT3*	*JAK2*	*MYC*	*RB1*	***TP53***
BRAF	***ERBB2***	*FOXL2*	*JAK3*	*MYCN*	***RET***	*VHL*
CCND1	*ERBB3*	*GNA11*	*KDR*	*NOTCH1*	*RHOA*	
CCNE1	*ERBB4*	*GNAQ*	***KIT***	*NPM1*	***ROS1***	
CDH1	***ESR1***	*GNAS*	***KRAS***	***NRAS***	***SMAD4***	
B. Genes on the Archer LIQUIDPlexTM ctDNA 28 Kit						
ALK	***CTNNB1***	***ESR1***	***IDH2***	*MAP2K2*	*NTRK3*	***ROS1***
AKT1	***DDR2***	***FGFR1***	***KIT***	***MET***	***PIK3CA***	***SMAD4***
AR	***EGFR***	***HRAS***	***KRAS***	***NRAS***	***PDGFRA***	*MTOR*
BRAF	***ERBB2***	***IDH1***	***MAP2K1***	*NTRK1*	***RET***	***TP53***

* genes that are common on both panel are highlighted with bold in grey cells

3.3. Mutation analysis of cfDNA from plasma samples

Eleven plasma samples were derived from CRC patients with no metastasis (68.7%), and 5 (31.3%) samples from patients with metastases at the diagnosis. Seven patients exhibited mutations in at least one of the 28 genes covered by the panel. Important to note that cfDNA-s were isolated from the same volume of plasma in each plasma sample and analyzed using the Archer LIQUIDPlexTM ctDNA 28 Kit. The cfNDA concentration was significantly higher in patients' plasma with metastasis compared to cfDNA concentration of patients with primary tumors ($p = 0.036$) (Fig. 2).

Based on our analyses, 7 plasma cfDNA (43.8%) harbored 13 mutations in 5 genes (*TP53*: 5 samples; *KRAS*: 4 samples; *NTRK3*: 2 samples; *SMAD4*: 1 sample; *BRAF*: 1 sample); cfDNA obtained from 9 plasma samples (56.2%) did not show any mutations. The summary of the sequencing results and the number of gene mutations and variants are summarized on Fig. 3.

Although only one-third of the samples were from patients with metastatic tumors, the majority of the mutations were associated with metastasis, further highlighting that the tumor genome is becoming increasingly unstable and mutations accumulate as the tumor progresses. Most of the alterations were missense mutations (n = 11, 84.6%), only two were nonsense mutations (15.4%). The highest variant allele frequency was observed in the *KRAS* gene (sample P18) with a 0.7879 value.

The detailed description of the mutations with genomic location and clinical significance of the cfDNA samples are shown in Supplementary Table 2.

3.4. Comparison of mutation patterns in tumor tissues and plasma samples obtained from the same patient

In order to test the similarity between the mutational pattern found in the same patient's tumor sample and cfDNA sample, a comparison was made in case of 9 patients. The Archer® VariantPlex® Solid Tumor 67 and LIQUIDPlexTM ctDNA 28 Kit panels contain 23 overlapping genes, but unfortunately not from each exon as shown in Fig. 4.

The mutational profile of matched tumor tissues and plasma samples (samples originated from the same patient) that were analyzed on the solid tumor and liquid biopsy panels separately are summarized in Table 4.

In one patient (P45) neither the primary tumor tissue nor the plasma cfDNA showed any mutations on the tested panels. Seven primary tumor

Table 2
Clinicopathological characteristics of patients from whom tumor and/or plasma samples are derived.

Sample	Sex	Age (year)	Localisa-tion	Tumor size (cm)	pTNM	Histological subtype	Grade	Aster-Coller stage	Ulce-ration	Representation of tumor cells in the tissue (%) a	cfDNA (ng/ul)
Both tumor DNA and plasma cfDNA samples are available from the same patients											
P21	M	63	colon	5	pT3 pN1b	adenocarc.	G2	n.a.	yes	80%	0.59
P28	F	56	rectosigma	6.5	pT2 pN0	adenocarc.	G2	n.a.	no	80%	0.59
P39	F	82	coecum	2.2	pT2 pNx	adenocarc.	G2	n.a.	no	80%	0.52
P40	F	86	colon	4.8	pT3 pN0	adenocarc.	G2	n.a.	yes	50%	0.40
P45	M	67	rectosigma	3.8/2.8	pT2 pN2a	duplex adenocarc. rectum/colon	G2	C1	yes	70%	1.52
P49	M	56	rectosigma	3.9	pT2 pN0	adenocarc.	G2	n.a.	no	80%	0.46
P50	F	58	sigma + distalis resectios	4.5	pT2 pN0	adenocarc.	G2	B1	yes	80%	2.85
P51	F	55	rectosigma	7.1	pT2 pN0	adenocarc.	G2	B1	no	70%	5.22
M33	M	72	liver	Nr	nr	metastatic carcinoma	nr	nr	nr	90%	4.31
Only plasma cfDNA samples are available											
P1	M	80	colon	5	pT3 pN2a pMx	adenocarc.	G2	C1	no	n.a.	5.73
P18	F	72	sigmoid	5	pT3 pN2a	adenocarc.	G3	C2	yes	30%	7.52
P20	F	60	rectosigma	4	pT3 pN1b pMx	adenocarc.	G3	C1	yes	30%	2.50
M37	F	50	liver	Nr	nr	adenocarc. metastaticum	nr	nr	nr	60%	24.90
M47	M	67	liver	Nr	nr	adenocarc. metastaticum	nr	nr	nr	40%	2.07
M52	M	75	liver	Nr	nr	neuroendocrin carcinoma	nr	nr	nr	40%	7.24
M58	M	69	liver	Nr	nr	adenocarc. metastaticum hepatis	nr	nr	nr	30%	17.3
Only primary tumor samples are available											
P16	M	60	colon	16	pT3 pN1b pMx	adenocarc.	G3	C1	yes	70%	0.15
P19	M	68	colon	8	pT2 pN1c	adenocarc.	G2	C1	yes	80%	0.29
P24	F	64	rectosigma	3.5	pT3 pN0	adenocarc.	G2	B2	no	70%	0.2
P26	M	66	rectosigma	4.5	pT3 pN2a	adenocarc.	G2	B2	yes	50%	0.11
P27	F	68	rectosigma	4	pT2 pN0	adenocarc.	G2	n.d.	no	80%	0.33
P46	M	65	colon	5.5	pT2 pN0	adenocarc.	G2	n.d.	no	80%	0
P64	M	77	colon	3	pT2 pN1a	adenocarc.	G2	n.d.	no	80%	0

Abbreviations: P: primary tumor; M: tumor metastasis; pTNM: primary tumor TNM stage; cfDNA: plasma cell free DNA; adenocarc.: adenocarcinoma; n.a.: not available; nr: not relevant; [a]representation of tumor cells in the corresponding tumor tissue.

tissues harbored mutations on the TP53, APC, KRAS, MET, KDR, NTRK3 and JAK3 genes. The TP53 gene was altered in 6 tumor tissues, but positions of the mutations were different within the genes. Using the LIQUIDPlexTM ctDNA 28 panel we found mutations in four cfDNA sample (P39, P49, P51 and M33), however only one (M33) had the same mutation also in the tumor tissue. Three genes, KRAS, P53 and the SMAD4 were mutated at the same positions in the tumor tissue and plasma cfDNA. Interestingly, one of the TP53 mutations (NP_000537.3: p.Arg306Ter) was only observed in cfDNA, but could not be detected in the primary metastatic tumor tissue (M33), even though genes and exons overlap in both panels. Other exciting observation on the samples of the P39 patient was that the position of the mutations in the KRAS gene were different in the solid tumor DNA and plasma cfDNA samples. It is important to note that in 8 cases the mutations were not covered by the panels, but more importantly in 7 cases although the gene and exon showed overlap in both panels, no mutations were detected.

4. Discussion

Currently, there is a wide range of cfDNA tests that can be used to investigate the gene mutations that are characteristic of various cancer types [19]. The emergence of liquid biopsy in the clinical setting is essential and beneficial for the future. Besides its many advantages, such as being minimally invasive, helping in the detection and monitoring of disease and response to therapy, it is important to note that the analysis is a multi-step process, with many technical difficulties and not yet standardized [14,20,21]. Despite our growing knowledge about the molecular alterations in different tumor types, including colorectal cancer, there are very few studies available to compare the mutation patterns between blood cfDNA and CRC tissue samples originated from the same patients. This approach helps to define the diagnostic and predictive value of mutations/alterations detected in the liquid biopsy samples [20].

In this study our aim was to use commercially available gene panels

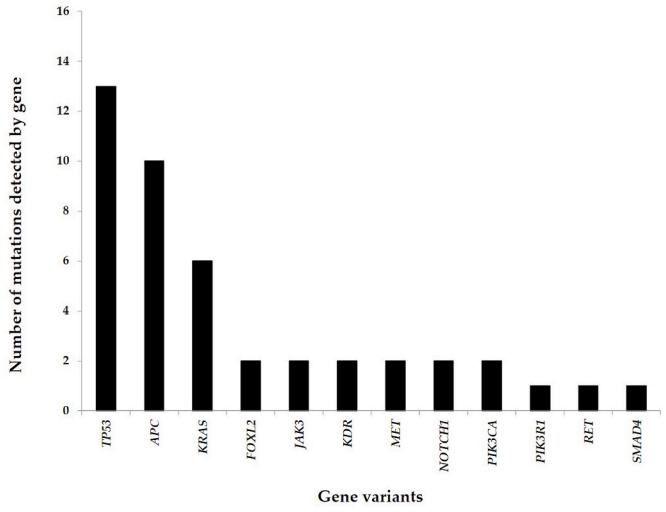

Fig. 1. Number of mutations detected by genes on the Archer® VariantPlex® Solid Tumor Panel in CRC samples. Ten of the 16 patients were found to have mutations in at least one of the 67 genes covered by the panel.

(Archer® VariantPlex® Solid Tumor Kit and LIQUIDPlexTM ctDNA 28 Kit) to detect the mutational status single nucleotide variants (SNVs), copy number variations (CNVs) and insertions/deletions (indels) of commonly altered genes associated with colorectal cancer and to verify

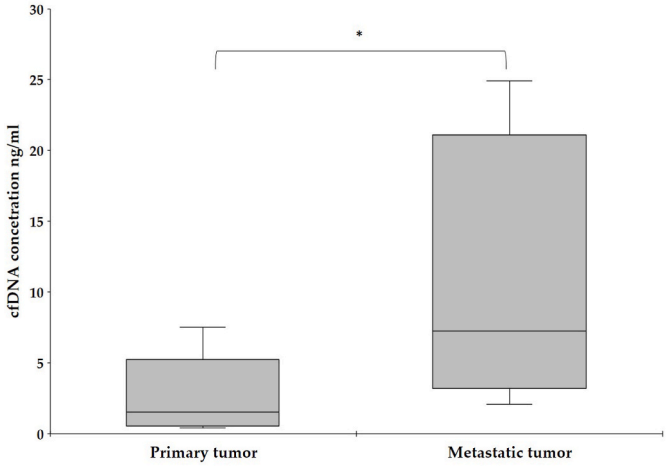

Fig. 2. Concentration distribution of cfDNA (ng/ml) isolated from plasma samples of patients with primary and metastatic colorectal tumors ($p = 0.036$).

Table 3
Relationship between the number of gene alterations in CRC tissues and clinical parameters of patients.

		Genes								
Characteristics	**No. of patients**	*TP53*	*APC*	*KRAS*	*FOXL2*	*JAK3*	*KDR*	*MET*	*NOTCH1*	*PIK3CA*
All patients	16	10	8	6	2	2	2	2	2	2
Number of mutations		13	10	6	2	2	2	2	2	2
Gender										
Female	7	4	2	3	1	1	1	1	1	1
Male	9	6	6	3	1	1	1	1	1	1
Age (years)										
<60	4	3	2	0	0	0	1	0	0	0
⩾60	12	7	6	6	2	2	1	2	2	2
Primary Tumor site										
Colon	8	6	4	5	1	2	0	2	0	1
Left colon	2	2	1	0	0	1	0	1	0	0
Right colon	6	4	3	3	1	1	0	1	0	1
Rectum	7	4	3	2	1	0	2	0	2	1
Size of primary tumour (cm)										
<5	9	6	3	3	1	1	2	1	2	1
⩾5	6	4	4	2	1	1	0	1	0	1
Primary tumor stage										
I-II	9	6	4	3	1	2	2	2	1	1
III	6	4	3	2	1	0	0	0	1	1
IV	0	0	0	0	0	0	0	0	0	0
No. of nodes in primary tumors										
N0 (0)	9	6	4	3	1	2	2	2	1	1
N1 (1-3)	4	3	2	2	1	0	0	0	0	1
N2 (>3)	2	1	1	0	0	0	0	0	1	0
Depth of invasion in primary tumors										
T1	0	0	0	0	0	0	0	0	0	0
T2	10	6	5	3	0	1	1	2	0	1
T3	5	4	2	2	**2**	1	1	0	**2**	1
T4	0	0	0	0	0	0	0	0	0	0

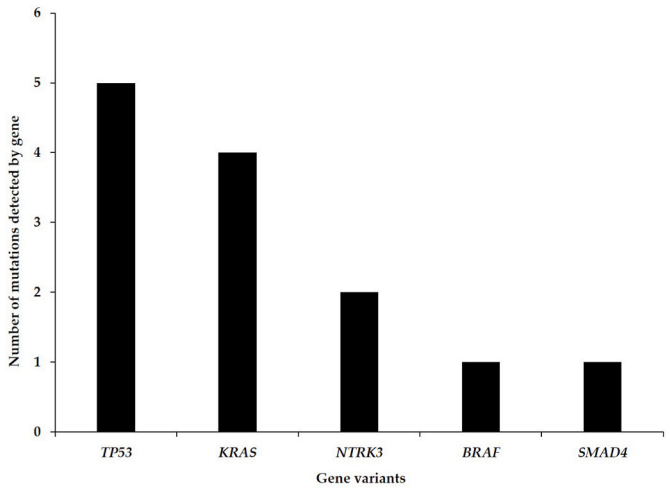

Fig. 3. Number of mutations identified by LIQUIDPlexTM ctDNA 28 panel in CRC samples in the *TP53, KRAS, NTRK3, BRAF* and *SMAD4* genes. Seven patients exhibited mutations in at least one of the 28 genes covered by the panel.

the mutation overlap between liquid biopsy and abnormalities detected in the tumor tissue. The two panels have an overlap in 23 genes, which enables a comparative analysis of the solid tumor and plasma cfDNA mutational status. The colorectal tumor samples showed a mutation pattern consistent with the published data. Using NGS the most frequently observed mutation in our samples was identified in the *TP53* gene, which is known to play a key role in the transformation of adenoma into adenocarcinoma and is found in 40–50% of sporadic CRCs. The second frequently mutated gene was *APC*, which leads to the activation of the WNT Wnt signaling pathway and it is the first step in tumorigenesis in this tumor type [22–25]. *KRAS* was the third frequently mutated gene, which acts as a driver gene in several tumors, as it plays an important role in the regulation of cell proliferation through the PI3K and MAPK pathways [26,27]. Mutations have also been found in the following genes *FOXL2, JAK3, KDR, MET, NOTCH1, PIK3CA, PIK3R1, RET, SMAD4*. Moreover, we identified two genes (*NOTCH1* and *FOXL2*) in association with advanced tumor stage. Dysregulation of the Notch1 receptor has been shown to facilitate the development and progression of colorectal cancer and identified as an independent predictor of disease progression [28]. However, mutation of *FOXL2* in association with tumor stage was not reported before, which can also contribute to the prediction of CRC progression.

Investigating the plasma samples, we also detected the main driver genes involved in the development and progression of CRC, which were covered by the LIQUIDPlexTM ctDNA panel, even though 56% of the samples did not show any mutations at all on this panel. It is important to note that the isolated cfDNA concentration was significantly higher in the plasma samples of patients with metastasis compared to cfDNA of patients with primary tumors in harmony with the findings that cfDNA levels and tumor aggressiveness are correlated in many types of tumors, including CRC [29–31]. Moreover, since cfDNA levels are significantly associated with overall survival and progression free survival, some have urged its potential clinical use for its simplicity and cheapness [32].

One of the main goals of our study was not only to examine gene mutations in tumor and plasma samples separately but also to select matched tissue and plasma samples from the same patient and compare the mutational status to detect the similarities and divergence between them. Overlapping the two mutational panels resulted in a total of 56

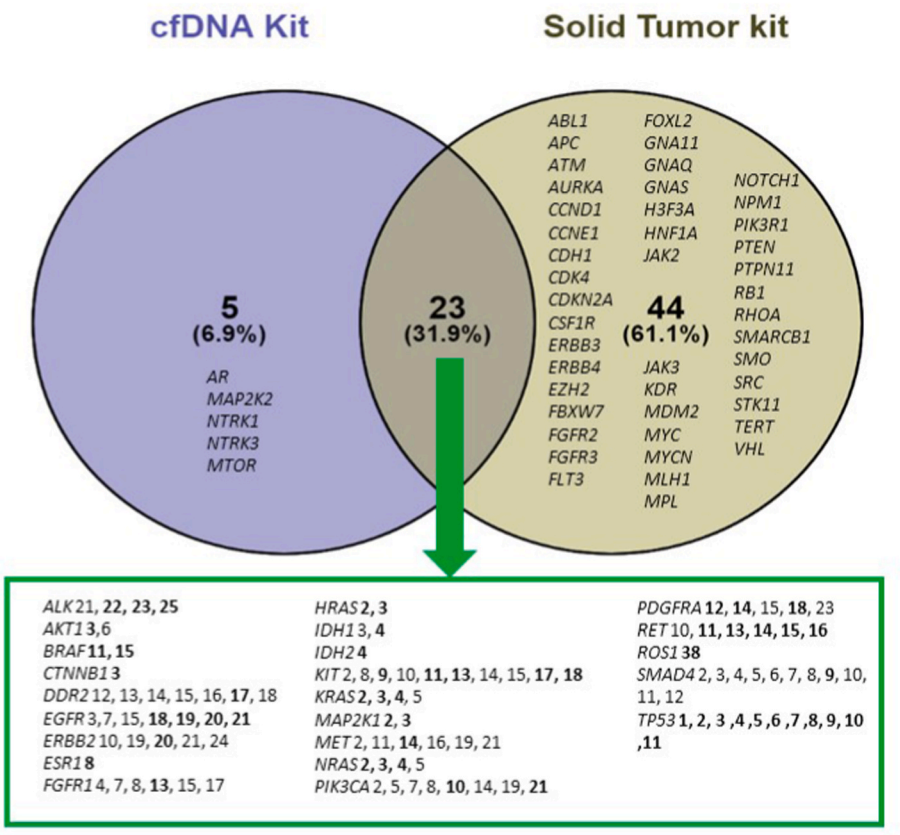

Fig. 4. Comparison of the gene composition of the Archer® VariantPlex® Solid Tumor 67 and LIQUIDPlexTM ctDNA 28 Kit panels. Venn diagram shows and the green arrow points the common genes on the two panels (23 genes). The numbers represent the exons being studied and the numbers in bold indicate the exons sequenced.

Table 4
Summary of mutational profiles of matched tumor and plasma samples detected on the Archer®VariantPlex® Solid Tumor 67 and on the LIQUIDPlexTM ctDNA 28 Kit panels.

Sample	Gene	TUMOR sample HGVSp	PLASMA sample HGVSp
P21	*TP53*	NP_000537.3:p.Gly245Ser	not detected
P28	*APC*	NP_000029.2:p.Glu1397Ter	not covered by the panel
	TP53	NP_000537.3:p.Ile195Thr	not detected
P39	*APC*	NP_000029.2:p.Arg1450Ter	not covered by the panel
	APC	NP_000029.2:p.Tyr935Ter	not covered by the panel
	KRAS	NP_004976.2:p.Gly12Asp	NP_004976.2:p.Ala59Thr
	MET	NP_000236.2:p.Thr992Ile	not detected
	TP53	NP_000537.3:p.Arg273His	not detected
P40	*JAK3*	NP_000206.2:p.Val722Ile	not covered by the panel
P45	-	–	–
P49	*APC*	NP_000029.2:p.Leu1129Ser	not covered by the panel
	APC	NP_000029.2:p.Arg805Ter	not covered by the panel
	KDR	NP_002244.1:p.Cys482Arg	not covered by the panel
	TP53	NP_000537.3:p.Leu194Arg	not detected
	NTRK3	not covered by the panel	NP_001012338.1:p.Val704Ile
P50	*TP53*	NP_000537.3:p.Arg273Cys	not detected
P51	*NTRK3*	not covered by the panel	NP_001012338.1:p.Val704Ile
M33	*APC*	NP_000029.2:p.Glu1451LysfsTer22	not covered by the panel
	*KRAS**	NP_004976.2:p.Gly12Cys	NP_004976.2:p.Gly12Cys
	*SMAD4**	NP_005350.1:p.Arg361Gly	NP_005350.1:p.Arg361Gly
	*TP53**	NP_000537.3:p.Arg282Trp	NP_000537.3:p.Arg282Trp
	TP53	not detected	NP_000537.3:p.Arg306Ter

Not detected: the gene, exon shows overlap in both panels, but no mutations were detected; not covered by the panel: the genes or exons are not covered by the panel; *same mutations were detected on both panels in the tumor DNA and cfDNA; HGVSp: protein variant nomenclature recommended by the Human Genome Variation Society.

exons in 23 genes. In addition to the important driver mutations (*P53, KRAS, APC*) discussed above, we also found alterations in the *MET* gene, which was not detected by the plasma panel despite the mutation being in the overlapping exon 14, and often found in CRC patients and it was also reported as a germline inherited risk factor for familial colorectal cancer [33]. Mutations of another two genes (*JAK3* and a *KDR*) were seen in two tissue samples (P40 and P49), but those were not part of the cfDNA panel. In contrast, mutant versions of the *NTRK3* gene were found in two plasma samples (p49, p51), but this gene was not present in the solid tumor panel. This gene is a tumor suppressor gene that is inactivated in colorectal cancer by both epigenetic and genetic mechanisms, and its function in CRC depends on the expression status of its ligand NT-3 [34]. Importantly, in a metastatic sample (M33) 4 genes (*APC, KRAS, SMAD4, TP53*) were mutated in the tumor tissue and three mutations (*KRAS, SMAD4, TP53*) were totally overlapped with the M33 plasma cfDNA sample. The *APC* gene was not a target gene on the cfDNA panel. However, interestingly, the plasma cfDNA of this patient revealed mutation of the *TP53* gene (NP_000537.3:p.Arg306Ter) that was not detected by the solid tumor panel, even though the genes and exons overlap in both panels. However, interestingly we found a TP53 gene mutation in one of the metastatic patients (M33) plasma sample, that was not detected on the solid tumor panel. This may represent heterogeneity within the tumor, but it may also represent an undetected metastasis within the same patient consisting of cells with different mutations. cfDNA analysis could inform about clonal heterogeneity and subclonal changes in real time highlighting mutations that are not detected in the primary tumor might be a sign for hidden metastasis. In this case cfDNA can replace the solid tumor biopsy when the latter is not feasible. This assumption is in agreement with published results [35–37].

We would like to highlight that 10 mutations that were detected in the matched tissues by the Archer®VariantPlex®Solid Tumor kit, were also covered by the cfDNA panel, however only three were found in the paired plasma cfDNA. Based on this observation, the sensitivity to characterize the mutations of the tumor tissue from the liquid biopsy is around 30%. Our comparative results between paired solid tumor samples and plasma cfDNA underline the importance of studying the matched solid tumor and plasma samples together to get a more comprehensive picture of the current state of the disease. Moreover, cfDNA analysis helps to identify heterogeneous tumor cell subpopulations that may be responsible for metastasis, consequently require different treatment [38].

We are aware that there are limitations to our study, one of which is that there is no overlap between the two panels for the *APC* gene, one of the most common driver genes in CRC. But in the other two most commonly mutated genes *TP53* and *KRAS* there is an almost complete overlap, so it was an adequate basis to define what percentage of mutational overlap we can expect between tumor and plasma samples obtained from the same patients.

We agree with Schara et al. who pointed out in a systematic review that based on the currently available literature data, no major conclusions can be drawn about the potential of cfDNA/ctDNA to diagnose and predict outcomes in colorectal cancer. The prognostic value of cfDNA and ctDNA presurgery is still unclear. The authors recommend that dedicated studies that primarily focus on cfDNA/ctDNA before surgery in colorectal cancer patients are needed [39].

Author contributions

Data curation, István Szász; Formal analysis, István Szász, Timea Kiss, Viktória Koroknai and Krisztina Jámbor; Funding acquisition, Róza Ádány and Margit Balázs; Investigation, Timea Kiss, Attila Mokánszki, Viktória Koroknai and János Deák; Methodology, István Szász, Timea Kiss, Attila Mokánszki, Viktória Koroknai, János Deák, Vikas Patel, Krisztina Jámbor and Margit Balázs; Resources, Róza Ádány and Margit Balázs; Supervision, Margit Balázs; Writing – original draft, István Szász and Attila Mokánszki; Writing – review & editing, István Szász, Viktória Koroknai, Róza Ádány and Margit Balázs.

Institutional Review Board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board. All tumor samples were handled according to the rules and regulations by the University of Debrecen, with the approval of the Hungarian ethics committee (TUKEB 17876–2018/EKU).

Informed consent statement

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

Funding

This research was co-financed by the National Research Development and Innovation Fund (grant number K-112327 and K-135752), the European Regional Development Fund (GINOP-2.3.2-15-2016-00005), and the Hungarian Academy of Sciences (TK2016-78) and the Eötvös Loránd Research Network (TKCS-2021/32). New National Excellence Program of the Ministry for Innovation and Technology from the Source of National research, Development and Innovation Fund (UNKP-21-4-II-DE-361 and UNKP-21-4-II-DE-363).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2022.101888.

References

[1] F. Baidoun, et al., Colorectal cancer epidemiology: recent trends and impact on outcomes, Curr. Drug Targets 22 (2021) 998–1009, https://doi.org/10.2174/1389450121999201117115717.

[2] K.W. Jasperson, et al., Hereditary and familial colon cancer, Gastroenterology 138 (2010) 2044–2058, https://doi.org/10.1053/j.gastro.2010.01.054.

[3] C.E. Bailey, et al., Increasing disparities in the age-related incidences of colon and rectal cancers in the United States, 1975-2010, JAMA Surg 150 (2015) 17–22, https://doi.org/10.1001/jamasurg.2014.1756.

[4] E.M. Stoffel, C.C. Murphy, Epidemiology and mechanisms of the increasing incidence of colon and rectal cancers in young adults, Gastroenterology 158 (2020) 341–353, https://doi.org/10.1053/j.gastro.2019.07.055.

[5] C. Alix-Panabieres, K. Pantel, Circulating tumor cells: liquid biopsy of cancer, Clin. Chem. 59 (2013) 110–118, https://doi.org/10.1373/clinchem.2012.194258.

[6] S.A. Joosse, K. Pantel, Tumor-educated platelets as liquid biopsy in cancer patients, Cancer Cell 28 (2015) 552–554, https://doi.org/10.1016/j.ccell.2015.10.007.

[7] A.L. Zygulska, P. Pierzchalski, Novel diagnostic biomarkers in colorectal cancer, Int. J. Mol. Sci. 23 (2022), https://doi.org/10.3390/ijms23020852.

[8] J.D. Cohen, et al., Detection and localization of surgically resectable cancers with a multi-analyte blood test, Science 359 (2018) 926–930, https://doi.org/10.1126/science.aar3247.

[9] M. Del Re, et al., Understanding the mechanisms of resistance in EGFR-positive NSCLC: from tissue to liquid biopsy to guide treatment strategy, Int. J. Mol. Sci. 20 (2019), https://doi.org/10.3390/ijms20163951.

[10] H. Zhou, et al., Liquid biopsy at the frontier of detection, prognosis and progression monitoring in colorectal cancer, Mol. Cancer 21 (2022) 86, https://doi.org/10.1186/s12943-022-01556-2.

[11] K.H. Khan, et al., Longitudinal liquid biopsy and mathematical modeling of clonal evolution forecast time to treatment failure in the PROSPECT-C phase II colorectal cancer clinical trial, Cancer Discov. 8 (2018) 1270–1285, https://doi.org/10.1158/2159-8290.CD-17-0891.

[12] M. Gerlinger, et al., Intratumor heterogeneity and branched evolution revealed by multiregion sequencing, N. Engl. J. Med. 366 (2012) 883–892, https://doi.org/10.1056/NEJMoa1113205.

[13] T. Yamada, et al., Liquid biopsy for the management of patients with colorectal cancer, Digestion 99 (2019) 39–45, https://doi.org/10.1159/000494411.

[14] C. Alix-Panabieres, K. Pantel, Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy, Cancer Discov. 6 (2016) 479–491, https://doi.org/10.1158/2159-8290.CD-15-1483.

[15] S. Jahr, et al., DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells, Cancer Res. 61 (2001) 1659–1665.

[16] K.L. Spindler, et al., Cell-free DNA in healthy individuals, noncancerous disease and strong prognostic value in colorectal cancer, Int. J. Cancer 135 (2014) 2984–2991, https://doi.org/10.1002/ijc.28946.

[17] F. Mouliere, et al., Multi-marker analysis of circulating cell-free DNA toward personalized medicine for colorectal cancer, Mol Oncol 8 (2014) 927–941, https://doi.org/10.1016/j.molonc.2014.02.005.

[18] A. Mokanszki, et al., Novel RB1 and MET gene mutations in a case with bilateral retinoblastoma followed by multiple metastatic osteosarcoma, Diagnostics 11 (2020), https://doi.org/10.3390/diagnostics11010028.

[19] H. Osumi, et al., Clinical utility of circulating tumor DNA for colorectal cancer, Cancer Sci. 110 (2019) 1148–1155, https://doi.org/10.1111/cas.13972.

[20] F. Salvianti, et al., Circulating tumour cells and cell-free DNA as a prognostic factor in metastatic colorectal cancer: the OMITERC prospective study, Br. J. Cancer 125 (2021) 94–100, https://doi.org/10.1038/s41416-021-01399-6.

[21] B. Molnar, et al., Circulating cell-free nucleic acids as biomarkers in colorectal cancer screening and diagnosis - an update, Expert Rev. Mol. Diagn 19 (2019) 477–498, https://doi.org/10.1080/14737159.2019.1613891.

[22] H. Li, et al., Targeting the oncogenic p53 mutants in colorectal cancer and other solid tumors, Int. J. Mol. Sci. 20 (2019), https://doi.org/10.3390/ijms20235999.

[23] K. Otori, et al., Infrequent somatic mutation of the adenomatous polyposis coli gene in aberrant crypt foci of human colon tissue, Cancer 83 (1998) 896–900, https://doi.org/10.1002/(sici)1097-0142(19980901)83:5<896::aid-cncr14>3.0.co;2-q.

[24] M. Miyaki, et al., Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors, Cancer Res. 54 (1994) 3011–3020.

[25] S. Cottrell, et al., Molecular analysis of APC mutations in familial adenomatous polyposis and sporadic colon carcinomas, Lancet 340 (1992) 626–630, https://doi.org/10.1016/0140-6736(92)92169-g.

[26] W. Kolch, Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions, Biochem. J. 351 Pt 2 (2000) 289–305.

[27] E. Castellano, et al., Requirement for interaction of PI3-kinase p110alpha with RAS in lung tumor maintenance, Cancer Cell 24 (2013) 617–630, https://doi.org/10.1016/j.ccr.2013.09.012.

[28] A. Tyagi, A.K. Sharma, C. Damodaran, A review on Notch signaling and colorectal cancer, Cells 9 (2020), https://doi.org/10.3390/cells9061549.

[29] S.A. Leon, et al., Free DNA in the serum of cancer patients and the effect of therapy, Cancer Res. 37 (1977) 646–650.

[30] A.K. Boysen, et al., Cell-free DNA levels and correlation to stage and outcome following treatment of locally advanced rectal cancer, Tumour Biol 39 (2017), 1010428317730976, https://doi.org/10.1177/1010428317730976.

[31] C. Bedin, et al., Diagnostic and prognostic role of cell-free DNA testing for colorectal cancer patients, Int. J. Cancer 140 (2017) 1888–1898, https://doi.org/10.1002/ijc.30565.

[32] D. Fernandez-Garcia, et al., Plasma cell-free DNA (cfDNA) as a predictive and prognostic marker in patients with metastatic breast cancer, Breast Cancer Res. 21 (2019) 149, https://doi.org/10.1186/s13058-019-1235-8.

[33] D.W. Neklason, et al., Activating mutation in MET oncogene in familial colorectal cancer, BMC Cancer 11 (2011) 424, https://doi.org/10.1186/1471-2407-11-424.

[34] Y. Luo, et al., NTRK3 is a potential tumor suppressor gene commonly inactivated by epigenetic mechanisms in colorectal cancer, PLoS Genet. 9 (2013), e1003552, https://doi.org/10.1371/journal.pgen.1003552.

[35] M. Murtaza, et al., Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer, Nat. Commun. 6 (2015) 8760, https://doi.org/10.1038/ncomms9760.

[36] L. De Mattos-Arruda, et al., Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle, Ann. Oncol. 29 (2018) 2268, https://doi.org/10.1093/annonc/mdx804.

[37] M. Russano, et al., Liquid biopsy and tumor heterogeneity in metastatic solid tumors: the potentiality of blood samples, J. Exp. Clin. Cancer Res. 39 (2020) 95, https://doi.org/10.1186/s13046-020-01601-2.

[38] C. Kin, et al., Colorectal cancer diagnostics: biomarkers, cell-free DNA, circulating tumor cells and defining heterogeneous populations by single-cell analysis, Expert Rev. Mol. Diagn 13 (2013) 581–599, https://doi.org/10.1586/14737159.2013.811896.

[39] S.J. Schraa, et al., Cell-free circulating (tumor) DNA before surgery as a prognostic factor in non-metastatic colorectal cancer: a systematic review, Cancers 14 (2022), https://doi.org/10.3390/cancers14092218.