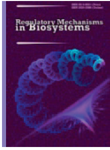

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Detection of postoperative recurrence in colorectal cancer: Exploring the value of liquid biopsy

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Recently, the number of publications concerning the use of liquid biopsy and, in particular, circulated tumor DNA (ctDNA) as well as the role of KRAS, BRAF, PIK3CA, and TP53 mutations for the diagnosis of early relapse in patients with colorectal cancer (CRC) has increased. However, it is necessary to accumulate a larger pool of data confirming its effectiveness and reliability for further implementation in clinical practice. The aim of this study was to determine the prevalence and impact of KRAS, BRAF, PIK3CA, and TP53 mutations on the survival of patients with stages I–III (CRC) using digital polymerase chain reaction (dPCR) and to evaluate the effectiveness of liquid biopsy in detecting ctDNA and predicting disease recurrence. 138 patients, scheduled for radical surgery for stage I–III CRC, were included in the study. DNA was extracted from tumor tissue samples and 3 plasma samples (obtained within 24 hours prior to surgery, 24 ± 1 hour and 90 ± 5 days after surgery) and then analyzed using dPCR for KRAS, BRAF, PIK3CA, and TP53 mutations. The dPCR was carried out using the QuantStudio 3D Digital PCR System (Applied Biosystems by ThermoFisher Scientific, USA) with the appropriate TaqMan Liquid Biopsy dPCR Assays (ThermoFisher Scientific, USA) (KRAS_512 (p.G12D), BRAF_476 (p.V600E), PIK3CA_775 (p.H1047R), and TP53_10662 (p.R248Q)). The studied mutations were found in 34 (24.6%) out of 138 tumor tissue samples. The prevalence of mutations in the study cohort was: KRAS – 9.4%, BRAF – 8.7%, PIK3CA – 3.6%, TP53 – 4.4%. The presence of at least one of the studied mutations significantly affected recurrence-free survival (RFS). The sensitivity of liquid biopsy for detecting ctDNA was 79.4%, and specificity was 100.0%. The recurrence rate was 59.3% for ctDNA-positive patients and 9.9% for ctDNA-negative patients. Liquid biopsy detected disease recurrence 2.14 months earlier than CT and colonoscopy. Ultimately, our findings support the role of liquid biopsy, particularly ctDNA, in advancing personalized treatment strategies for CRC.

Keywords: colorectal cancer; liquid biopsy; KRAS; BRAF; PIC3CA; TP53.

Introduction

The recurrence of colorectal cancer (CRC) following radical surgery remains a major factor significantly impacting patient survival (Balboa-Barreiro et al.,2024). Despite ongoing advancements in multimodal CRC treatment approaches, the declining recurrence rates observed during the 20th century have now plateaued (Tsai et al., 2024). Recurrence is diagnosed in approximately 30% of patients who have undergone radical treatment (Zheng et al., 2020). The median overall survival for patients with local recurrence is around 27 months, for those with distant metastases – 29 months, and for those with both – only 13 months (Boute et al., 2024).

Evaluating treatment efficacy and actively monitoring disease progression are essential for the early detection of asymptomatic recurrences. Such strategies aim to enhance the overall survival of patients with recurrent CRC. Similar to other tumor types, existing CRC diagnostic methods require further enhancement to address these challenges (Oh & Joo, 2020; Moskalenko et al., 2023).

Genetic mutations play a pivotal role in CRC recurrence (Ogunwobi et al., 2020). For instance, mutations in the KRAS and TP53 genes are linked to lymph node involvement, while mutations in the BRAF gene are associated with local disease recurrence. Furthermore, all these mutations are correlated with the development of distant metastases (Testa et al., 2020). Mutations in the KRAS, BRAF, PIK3CA, and TP53 genes may influence the tumor's sensitivity to prescribed therapies (Zhuang et al., 2021). As a result, identifying and regularly monitoring tumor-associated mutations have become the cornerstone of early CRC recurrence detection (Zhou et al., 2022).

Tumor tissue obtained via biopsy or following radical surgery is commonly used for genetic profiling (Scarlotta et al., 2019). However, throughout the tumor's lifespan, circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), tumor microRNA, proteins, and various other cellular fragments are released into the bloodstream. These can serve as materials for tumor genome analysis (Vacante et al., 2020). The technique used to identify tumor-associated fragments in blood or other biological fluids is known as liquid biopsy (Nikanjam et al., 2022). Unlike traditional tissue biopsy, this method is minimally invasive, making it a promising tool for monitoring during treatment and follow-up (Najafi et al., 2024).

ctDNA is considered one of the most promising biomarkers for liquid biopsy (Cescon et al., 2020). It constitutes a part of the cell-free DNA (cfDNA) pool, comprising DNA fragments generated through cell necrosis or apoptosis, originating from both somatic and tumor cells. Its short half-life, ranging from 15 minutes to several hours, enables real-time monitoring of residual disease (Bittla et al., 2023).

Recently, the number of publications concerning the use of liquid biopsy and, in particular, ctDNA for the diagnosis of early relapse in patients with CRC has increased. However, for the implementation of this method in clinical practice, it is necessary to accumulate a larger pool of data confirming its effectiveness and reliability (Tao et al., 2024).

The objective of our research is to determine the prevalence and impact of KRAS, BRAF, PIK3CA, and TP53 mutations on the survival of patients with stages I–III colorectal cancer (CRC) using digital polymerase chain reaction (dPCR) and to evaluate the effectiveness of liquid biopsy in detecting circulating tumor DNA (ctDNA) and predicting disease recurrence.

Materials and methods

Study design. The patient selection was conducted at the Sumy Regional Council Municipal Non-Profit Enterprise "Sumy Clinical Oncology Center" (SRC MNE SCOC), from December 2019 to December 2020. A total of 138 individuals, scheduled for radical surgery for stage I–III cancer of the colon or rectum, were included in the

study. Inclusion criteria were: Age over 18 years; Histologically confirmed diagnosis of CRC; Stage I–III of the disease; Compensated comorbidities; Signed informed consent to participate in the study. Exclusion criteria were: Age under 18 years; Metastatic stage of the disease; Decompensated comorbidities that alone or in combination with CRC could lead to patient death in the near term; Presence of another malignant tumor; Patient refusal to participate in the study.

The subsequent study was divided into several sequential phases. Two types of samples were analyzed during the first phase. First, tumor tissue samples in the form of paraffin-embedded blocks. Tumor DNA was extracted from these samples and analyzed using dPCR for KRAS, BRAF, PIK3CA, and TP53 mutations. Second, blood plasma samples, obtained within 24 hours prior to surgery. cfDNA was extracted from the plasma samples, and the presence of ctDNA was determined through the detection of KRAS, BRAF, PIK3CA, and TP53 mutations using dPCR.

Based on these analyses, patients were divided into two groups. The first group is the patients without any detected mutations in tumor tissue and no ctDNA in plasma samples. The second - patients with at least one mutation and/or ctDNA detected. Patients from the first group followed to the observation phase, while patients from the other group proceeded to the second phase of the study.

During the second phase, two venous blood samples were collected from patients. The first sample was taken 24 hours (± 1 hour) after surgery, the second – 90 days (± 5 days) post-surgery. Plasma sample analysis during this phase was performed similarly to the first phase. After this, patients from the second group also entered the observation phase. The study design is illustrated in Figure 1.

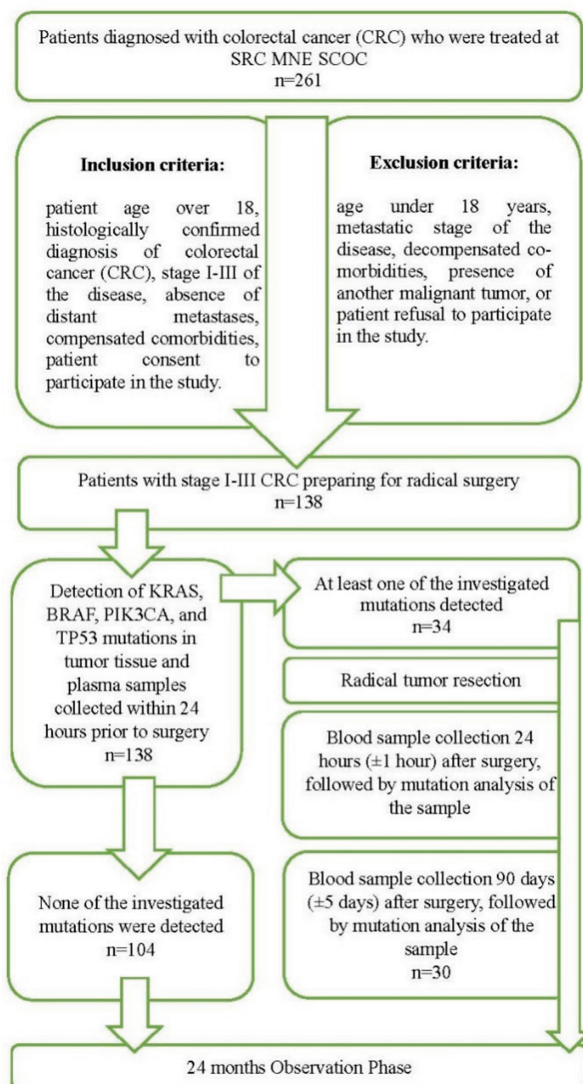

Fig. 1. Flowchart of the study design

The study was approved by the Bioethics Commission for the Conduct of Experimental and Clinical Research at the Medical Institute of Sumy State University (protocol No. 2/12, dated 10.12.2019). All patients included in the study signed informed consent prior to participation.

DNA extraction from tumor tissue. DNA was extracted from tumor tissue samples using the Quick-DNA FFPE Kit (Zymo Research, USA) according to the manufacturer's instructions. Before starting the procedure as per the manual, the lyophilized Proteinase K and lyophilized RNase were reconstituted, and the Genomic DNA Wash 2 buffer was prepared.

The process began with the preparation of 25 mg tumor tissue samples. For deparaffinization, excess paraffin was mechanically removed from the tissue sample. The sample was placed into a 1.5 mL microcentrifuge tube, and 400 μL of the deparaffinization solution provided in the kit was added. The tube was incubated at 55°C for 1 minute, and the deparaffinization solution was subsequently removed. To digest the tumor tissue, 45 μL of water, 45 μL of Digestion Buffer, and 10 μL of Proteinase K were added to the sample. The mixture was incubated at 55 °C for 1 hour, followed by an incubation at 94 °C for 20 minutes. Subsequently, 5 μL of RNase was added, and the sample was incubated at room temperature for 5 minutes.

For DNA purification, 350 μL of Genomic Lysis Buffer was added to the mixture, followed by 135 μL of isopropanol. The sample was centrifuged at 12,000 g for 1 minute in a Microspin 12 centrifuge (BioSan, Latvia). The supernatant was transferred to a Zymo-Spin IICR Column placed in a collection tube, and the sample was centrifuged at 10,000 g for 1 minute. The flow-through was discarded. The column was washed with 400 μL of Genomic DNA Wash 1 and centrifuged at 10,000 g for 1 minute, following which the flow-through was discardied. Next, 700 μL of Genomic DNA Wash 2 was added, and the sample was centrifuged at 12,000 g for 1 minute. This step was repeated with an additional 200 μL of Genomic DNA Wash 2. After completing the purification steps, the column was transferred to a clean microcentrifuge tube, and 50 μL of DNA Elution Buffer was added. The sample was incubated at room temperature for 5 minutes. The final step involved centrifugation at maximum speed for 30 seconds to elute the DNA.

Collection and preparation of plasma samples. The collection and preparation of plasma samples were performed according to the recommendations from the manual of the MagMax Cell-Free Nucleic Acid Isolation Kit (ThermoFisher Scientific, USA). Whole blood samples (4 mL) were collected into K2 EDTA vacuum tubes using a disposable BD Vacutainer system. Subsequently, the whole blood was centrifuged in an Eppendorf 5702R centrifuge (Eppendorf, Germany) at 2000 g for 10 minutes at 4 °C. The resulting plasma (~2 mL) was transferred to a new tube and centrifuged again at 6000 g for 30 minutes at 4 °C. The supernatant was then transferred into a clean tube. Plasma samples were frozen immediately after collection and stored at –20 °C in a Liebherr Comfort refrigerator (Liebherr, Germany). Further transport to the laboratory for DNA isolation was carried out within 30 minutes using a thermal bag with frozen cold packs.

Isolation of cfDNA from plasma. cfDNA was extracted from plasma samples using the MagMax Cell-Free Nucleic Acid Isolation Kit (ThermoFisher Scientific, USA). The procedure was performed according to the instructions provided by the manufacturer for manual extraction. Minor modifications were made to adapt the protocol to the available laboratory equipment. After thawing the plasma sample (2 mL) to room temperature for 5 minutes, 30 μL of Proteinase K and 1 mL of Lysis/Binding Solution were added. The mixture was incubated for 30 minutes at 65 °C with vortexing every 5 minutes. The sample was then cooled on ice for 5 minutes. For DNA binding to magnetic particles, 1.5 mL of Lysis/Binding Solution and 60 μL of thoroughly vortexed magnetic beads were added to the sample. The mixture was then mixed at maximum speed on a shaker for 20 minutes. Subsequently, the sample was placed on a magnetic stand (DynaMag-2) for 10 minutes (steps involving DynaMag-50 were omitted due to its absence), and the supernatant was removed.

To wash the magnetic particles bound to cfDNA, 1 mL of Wash Solution 1 was added and mixed by pipetting. The sample was placed back on the magnetic stand for 2 minutes or until the magnetic beads were fully settled, and the supernatant was discarded. The next step involved adding 1 mL of 80% ethanol, vortexing, and removing the supernatant (this step was repeated twice). Subsequently, all remaining liquid was removed, and the magnetic beads were dried for 5 minutes at room temperature. For cfDNA elution, 400 µL of Elution Solution was added to the magnetic beads, vortexed for 5 minutes at maximum speed, and the supernatant was transferred to a new tube. According to the manual, steps involving cfDNA concentration were performed by re-binding the DNA to magnetic beads, washing them, and re-eluting the cfDNA.

Digital polymerase chain reaction (dPCR). After DNA extraction, the concentration of DNA or cfDNA in the obtained samples was measured. The analysis was performed using the Qubit 4 Fluorometer (Invitrogen by ThermoFisher Scientific, USA) with the Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, USA) for DNA samples extracted from tumor tissue and the Qubit dsDNA HS Assay Kit (ThermoFisher Scientific, USA) for cfDNA samples extracted from plasma. The procedure followed the laboratory manual provided by the manufacturer. To 199 µL of the working solution from the respective kit, 1 µL of the sample was added, vortexed, and incubated at room temperature for 2 minutes.

The dPCR was carried out using the QuantStudio 3D Digital PCR System (Applied Biosystems by ThermoFisher Scientific, USA). The reaction mixture for dPCR was prepared by combining the sample, nuclease-free water, QuantStudio 3D Digital PCR Master Mix, and one of the TaqMan Liquid Biopsy dPCR assays (ThermoFisher Scientific, USA) according to the TaqMan assay instructions. Four different TaqMan panels were used to identify mutations: KRAS_512 (p.G12D), BRAF_476 (p.V600E), PIK3CA_775 (p.H1047R), and TP53_10662 (p.R248Q). Each panel included two types of fluorescent dyes: FAM and VIC. A fluorescent FAM signal (blue) indicated the presence of the mutation in the sample (amplification of mutant DNA), while the VIC signal (red) indicated DNA without the targeted mutation (wild-type DNA).

The reaction mixture was loaded onto QuantStudio™ 3D Digital PCR 20K Chips using the QuantStudio™ 3D Digital PCR Chip Loader. For each tumor tissue sample and each plasma sample collected within 24 hours before surgery, eight chips were prepared (two for each of the four mutations: one primary and one duplicate). For plasma samples collected after surgery, two chips were prepared per sample (one primary and one duplicate).

PCR amplification was performed using the ProFlex 2 × Flat PCR System (Applied Biosystems by ThermoFisher Scientific, USA). The thermal cycling protocol recommended in the manual was used: Stage 1: 95 °C for 10 minutes (ramp rate 2 °C/s), 1 cycle; Stage 2: 60 °C for 1 minute + 94 °C for 30 seconds (ramp rate 2 °C/s), 40 cycles; Stage 3: 98 °C for 10 minutes (ramp rate 2 °C/s), 1 cycle; Stage 4: 4 °C (ramp rate 1 °C/s), used as a hold stage.

The fluorescence signals described above were read from the chips using the QuantStudio™ 3D Digital PCR Instrument. This analyzer detects signals from each individual well of the chip. Chips with successful amplification in more than 16,000 wells were considered suitable for analysis. The obtained results were processed using the QuantStudio 3D AnalysisSuite Software.

The observation phase. The observation phase for patients lasted 24 months from the date of surgery and ended for the last participant in December 2022. In accordance with local practice standards, computed tomography (CT) of the chest, abdomen, and pelvis, as well as colonoscopy, were performed every six months. Local recurrence of the disease was confirmed using colonoscopy or CT. The presence of distant metastases was assessed based on CT data. The time interval from the date of surgery to the date of confirmed disease recurrence was considered recurrence-free survival (RFS).

Comparison of obtained results with TCGA. To compare the prevalence of selected mutations in the studied group and among of European individuals with CRC, the TCGA database (https://portal.gdc.cancer.gov) was used.

Statistical analysis. Data collection and systematization were performed using Microsoft Excel. For comparing categorical variables, the Pearson χ^2 test was used ($P < 0.05$). Visualization of survival curves for patients in groups with and without the studied mutations was performed using the Kaplan-Meier method. The presence of a statistically significant difference between these groups regarding recurrence probability was assessed using the Log-rank test ($P < 0.05$). The significance of the effect of the studied mutations on recurrence-free survival (RFS) was determined through Cox multivariate regression analysis with the Breslow method (95% confidence interval (CI), $P < 0.05$). A two-sample proportion test was used to compare the prevalence rates of the selected mutations in the study group and the TCGA database patients. Data analysis was conducted using Stata V.18.0 software (StataCorp, Texas, USA; www.stata.com; 2024).

Evaluation of liquid biopsy effectiveness. The effectiveness of liquid biopsy for detecting ctDNA was evaluated by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the method. The formulas used are provided in Table 1 (Hruzyeva et al., 2018).

Table 1
The method for evaluation of liquid biopsy effectiveness

Indicator	Formula
Sensitivity	a/(a+c) x100%
Specificity	d/(b+d) x100%
Positive predictive value (PPV)	a/(a+b) x100%
Negative predictive value (NPV)	d/(c+d) x100%
Accuracy	(a+d)/(a+b+c+d) x100%

Notes: a – true positive results, b – false positive results, c – false negative results, d – true negative results.

The effectiveness of liquid biopsy for predicting disease recurrence was evaluated based on the RFS indicator for ctDNA-positive and ctDNA-negative patients, as well as by the difference in time from ctDNA detection to disease recurrence confirmation using CT and colonoscopy.

Results

Patient characteristics. The study group consisted of 74 men and 64 women. The average age of the participants was 63 years (ranging from 34 to 83 years). The vast majority of patients had stage II–III cancer (127/138, 92.0%), with tumor localization in the rectum (74/138, 53.6%). The clinical and pathological characteristics of the study group are presented in Table 2.

Table 2
The clinical and pathological characteristics of the study group

Patient characteristics		Number of patients, n (%); N = 138
Age, years	range	34–83
	average	63
	<65	68 (49.3)
	≥65	70 (50.7)
Sex, n	men	74 (53.6)
	women	64 (46.4)
Tumor localization, n	colon	64 (46.4)
	rectum	74 (53.6)
Stage, n	I	11 (8)
	II–III	127 (92)
Category T, n	T1–2	12 (8.7)
	T3–4	126 (91.3)
Category N, n	N0	92 (66.7)
	N1–2	46 (33.3)
Tumour differentiation grade, n	G1	82 (59.4)
	G2–3	56 (40.6)
Neoadjuvant therapy, n	yes	58 (42)
	no	80 (58)
Adjuvant therapy, n	yes	74 (53.6)
	no	64 (46.4)

Prevalence of the studied mutations and their association with clinical and pathological characteristics of patients. According to the results of digital PCR, the studied mutations were identified in the

tumor tissue samples of 34 (24.64%) patients, including 12 (35.3%) patients with a KRAS gene mutation, 12 (35.3%) patients with a BRAF gene mutation, 4 (11.8%) patients with a PIK3CA gene mutation, 4 (11.8%) patients with a TP53 gene mutation, 1 (2.9%) patient with co-mutation in the KRAS and TP53 genes, and 1 (2.9%) patient with co-mutation in the PIC3CA and TP53 genes. The prevalence of mutations in the studied cohort was as follows: KRAS – 9.4%, BRAF – 8.7%, PIK3CA – 3.6%, TP53 – 4.4%. Figures 2 and 3 illustrate examples of identifying the presence of the KRAS mutation in the tumor tissue sample.

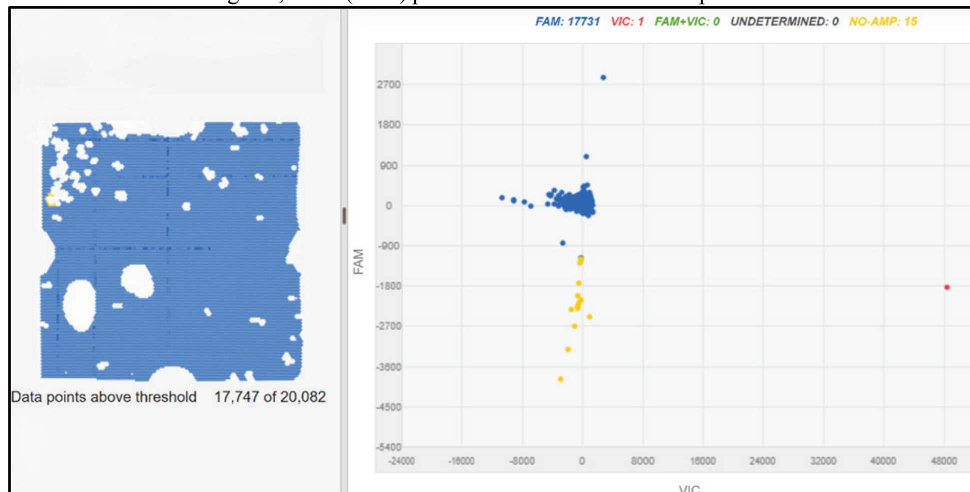

Fig. 2. Visualization of the tumor tissue sample study result with a mutation in the KRAS gene

Fig. 3. Visualization of the tumor tissue sample study result without a mutation in the KRAS gene

According to the results of the analysis of the relationship between the clinical-pathological characteristics of the patients and the presence of the studied mutations, no significant correlation was found (Table 3).

Assessment of the impact of mutations on RFS. Among the 138 patients in the study cohort, disease recurrence was recorded in 27 (19.6%) patients: 9 (33.3%) cases had tumors localized in the colon and 18 (66.7%) in the rectum. In 16 (59.3%) from 27 patients mutations were detected in tumor tissue samples: in the KRAS gene 5/16 (31.3%), BRAF 6/16 (37.5%), PIK3CA 2/16 (12.5%), and TP53 3/16 (18.7%). During the assessment of the impact of mutations on survival, it was found that patients without the studied mutations had better RFS (Log-rank P = 0.0001, Fig. 4).

According to the results of Cox regression analysis, age (P = 0.044) and mutations in the KRAS, BRAF, PIK3CA, and TP53 genes (P = 0.0001) were identified as independent predictors of RFS. Patients younger than 65 years and without these mutations had a better RFS (Table 4).

Comparison of the obtained results with TCGA. The TCGA electronic database contained 753 cases of identified mutations in tumor tissue samples from European descent patients with stage I–III colorectal cancer. According to the search results, the KRAS mutation was found in 81 (10.8%) of 753 cases, BRAF in 73 (9.7%), PIK3CA in 21 (2.8%), and TP53 in 27 (3.6%) of 753 cases. In the studied cohort, KRAS was found in 13 (9.4%) of 138 cases, BRAF in 12 (8.7%), PIK3CA in 5 (3.6%), and TP53 in 6 (4.4%) of 138 cases.

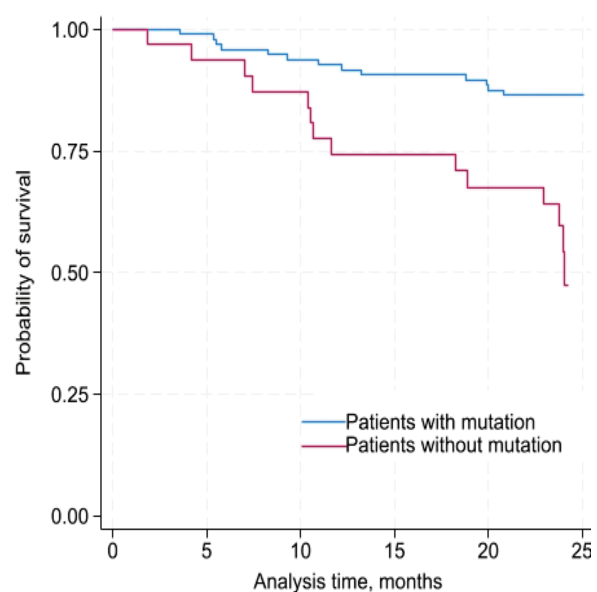

Fig. 4. Kaplan-Meier curves demonstrating RFS in radically operated CRC patients based on the results of molecular genetic profiling of the tumor

Table 3
Results of comparison of categorical variables to determine the relationship between clinical-pathological characteristics of the patient and the presence of the studied mutations

Patient characteristics	Total N = 138	With mutations N = 34	Without mutations N = 104	□²(P)
Age, n				
<65	68	13	55	1.9219 (0.166)
≥65	70	21	49	
Sex, n				
Men	74	19	55	0.0926 (0.761)
Women	64	15	49	
Tumor location, n				
Colon	64	15	49	0.0926 (0.761)
Rectum	74	19	55	
Stage, n				
I	11	4	7	0.8884 (0.641)
II	81	19	62	
III	46	11	35	
Category T, n				
T1	5	1	4	3.4344 (0.329)
T2	7	3	4	
T3	92	25	67	
T4	34	5	29	
Category N, n				
N0	92	23	69	0.1729 (0.917)
N1	43	10	33	
N2	3	1	2	
Tumour differentiation grade, n				
G1	82	17	65	1.6604 (0.198)
G2-3	56	17	39	
Neoadjuvant therapy, n				
Yes	58	14	44	0.0135 (0.908)
No	80	20	60	
Adjuvant therapy, n				
Yes	74	17	57	0.2382 (0.626)
No	64	17	47	

Table 4
Results of Cox regression analysis to determine factors affecting RFS

Patient characteristics	RFS hazard ratio (CI – 95%), P
Age (<65 vs ≥65)	2.44 (1.02–5.78), 0.044
Sex (men vs women)	1.90 (0.81–4.47), 0.142
Tumor location (colon vs rectum)	1.42 (0.38–5.30), 0.601
Stage (I vs II–III)	16.70 (1.92–2.47), 0.152
Category T (T1–2 vs T3–4)	0.90 (0.21–4.42), 0.842
Category N (N0 vs N1–2)	1.74 (0.74–4.08), 0.203
Tumour differentiation grade (G1 vs G2–3)	1.36 (0.60–3.10), 0.461
Neoadjuvant therapy (yes vs no)	1.25 (0.81–4.47), 0.604
Adjuvant therapy (yes vs no)	3.18 (0.88–11.50), 0.078
Presence of studied mutations (yes vs no)	4.13 (1.89–9.00), 0.0001

The prevalence of selected mutations between patients of the study group and those of the TCGA database was not significantly different: KRAS 9.4% vs 10.8% (P = 0.8700), BRAF 8.7% vs 9.7% (P = 0.9131), PIK3CA 3.6% vs 2.8% (P = 0.9244), TP53 4.4% vs 3.6% (P = 0.9256, Fig. 5).

Evaluation of liquid biopsy effectiveness. ctDNA in plasma samples collected within 24 hours before surgery was detected in 27 (19.6%) patients. The mutations found in the plasma samples completely matched those identified in the tumor tissue. Based on these data, the sensitivity of the method for detecting ctDNA in plasma samples was 79.4% (27 patients out of 34). Since no false-positive cases were observed, the specificity of the method was 100%. The PPV was 100%, and the NPV was 50%. The method's accuracy was 82.9%. The recurrence rate rate for ctDNA-positive patients was 59.3% (relapse occurred in 16 out of 27 patients), while for ctDNA-negative patients – 9.9% (relapse occurred in 11 out of 111 patients).

CtDNA in plasma samples collected 24 hours (± 1 hour) after surgery was detected in only 2 (5.9%) of 34 samples. At the timepoint of blood sample collection 90 days (± 5 days) after surgery, 4 patients refused to provide blood samples. Therefore, only 30 patients' samples were tested instead of 34. Of the 30 samples tested, ctDNA was detected in 7 (23.3%) cases, 2 of which were identified earlier in the blood samples collected 24 hours (± 1 hour) after surgery. Detailed information about these patients is provided in Table 5.

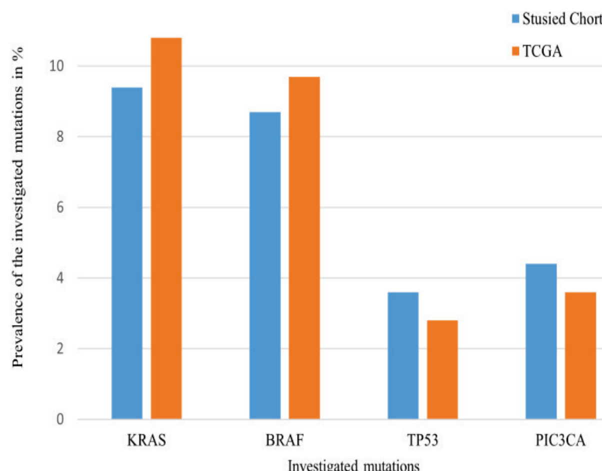

Fig. 5. Comparison of the prevalence of mutations in the KRAS, BRAF, PIK3CA, and TP53 genes between the study group and the TCGA database

Table 5
Characteristics of patients with ctDNA or reappearance of ctDNA in samples collected after surgery

Patient study number	Time of ctDNA detection after surgical intervention	Gene mutation	Time from surgical intervention to recurrence confirmation (months)	Time from detection of cfDNA to recurrence confirmation (months)	Recurrence (yes(type)/no)
19	24 hours and 90 days after (3 months)	KRAS	1.83	1.83	yes (liver metastasis)
121	24 hours and 90 days after (3 months)	KRAS	4.17	4.17	yes (local recurrence)
30	90 days after (3 months)	BRAF	10.53	7.53	yes (local recurrence)
45	90 days after (3 months)	BRAF	7.03	4.03	yes (local recurrence)
83	90 days after (3 months)	KRAS	7.47	4.47	yes (local recurrence)
99	90 days after (3 months)	PIK3CA and TP53	10.67	7.67	yes (lung metastasis)
128	90 days after (3 months)	TP53	10.43	7.43	yes (liver metastasis)

Thus, the average time from the detection of ctDNA in plasma to disease recurrence was 5.3 months. For the same patients, the average time from surgery to the confirmation of progression by CT and colonoscopy was 7.45 months. Therefore, liquid biopsy allowed us to predict disease recurrence 2.15 months earlier than its actual diagnosis.

Discussion

In this study, we investigated the prevalence of KRAS, BRAF, PIK3CA, and TP53 mutations in a cohort of patients with stages I–III CRC using dPCR and their impact on RFS. It has been determined that the presence of at least one of the investigated mutations leads to the worsening of RFS.

Each of the investigated mutations have a significant role in carcinogenesis, CRC progression, and the development of treatment resistance (Sclafani et al., 2020). The oncogene KRAS encodes a protein belonging to the RAS family. This protein plays a critical role in

signal transduction in cells and regulates processes such as proliferation, differentiation, migration, angiogenesis, and apoptosis. Under normal conditions, it is activated by external signals and initiates one of two signaling pathways: MAPK/ERK, responsible for regulating the cell cycle, or PI3K/AKT, responsible for cell survival. Once the external signal subsides, the protein "switches off." In the case of a mutation, the protein loses its deactivation ability, leading to disruption of normal cell growth control (Uprety et al., 2020). The clinical significance of this mutation is associated with poorer disease prognosis and the development of resistance to EGFR inhibitors, such as cetuximab and panitumumab (Li et al., 2020; Zhou et al., 2021). In our study, KRAS mutations were found in 9.42% of CRC patients, which correlates with the data from the TCGA database.

The oncogene BRAF encodes the B-raf protein, which plays a key role in signal transmission through the MAPK/ERK pathway. Under normal conditions, this protein regulates the cell cycle, differentiation, and cell adaptation under stress conditions. A mutation in BRAF causes the disruption of these functions, resulting in uncontrolled cell division (Śmiech et al., 2020). Like KRAS mutation carriers, BRAF mutation-positive patients demonstrate poor response to EGFR inhibitors (Li et al., 2020; Zhou et al., 2021). Despite this, BRAF remains a key target for targeted therapy, specifically BRAF inhibitors (dabrafenib, encorafenib, plixorafenib) (Maji et al., 2024; Yaeger et al., 2024). The prevalence of BRAF mutations in CRC patients, according to published studies, ranges from 2% to 12% (Ciombor et al., 2022; Kopetz et al., 2024), which is consistent with the findings of our current study.

The oncogene PIK3CA encodes the catalytic subunit of the PI3K protein, which plays a key role in various cellular processes by activating the PI3K/AKT/mTOR signaling pathway. This pathway regulates cell growth, proliferation, and metabolism. PI3K/AKT stimulates glucose uptake, protein and lipid synthesis, adapting the cell to metabolic needs, and ensures cell survival under stress conditions, such as nutrient or oxygen deprivation. A mutation in the PIK3CA gene leads to hyperactivation of this signaling pathway, which contributes to uncontrolled cell growth, increased tumor cell viability, and resistance to apoptosis (Voutsadakis et al., 2021). Like BRAF, PIK3CA mutations are targeted for therapy, with PI3KCA inhibitors (alpelisib, umbralisib) (Vanhaesebroeck et al., 2021). In our study, the prevalence of PIK3CA mutations in CRC patients was 3.62%. This result was consistent with the data from the TCGA database.

The oncogene TP53 encodes the p53 protein, which is a central regulator of anti-cancer mechanisms in cells. This protein is often called as the "guardian of the genome" due to its role in controlling the cell cycle and DNA repair. Additionally, p53 triggers the apoptosis mechanism through activation of BAX and PUMA genes in the case of irreversible DNA damage, controls cell senescence (aging), prevents the transformation of cells into tumor cells, and suppresses angiogenesis, limiting tumor growth (Feroz et al., 2020). A mutation in the TP53 gene disrupts the described functions of p53 and leads to uncontrolled division of tumor cells and treatment resistance. Moreover, the presence of TP53 mutation is associated with resistance to radiation therapy (Chen et al., 2022). The prevalence of TP53 mutations in our cohort of patients was 4.35%. This result was also consistent with the data from the TCGA database.

In addition to studying described mutations, we assessed the effectiveness of liquid biopsy for detecting ctDNA and predicting CRC recurrence in patients after radical resection of the tumor. We found that the sensitivity of liquid biopsy for detecting ctDNA was 79.4%, and the specificity was 100%. Mas et al. (2019) in their study identified ctDNA for detecting BRAF mutations in 425 CRC patients using dPCR and next-generation sequencing (NGS). Their sensitivity was 76.7%, with a specificity of 98.9%. Chung et al. (2024) Chung evaluated the performance of a commercial ctDNA testing system for detecting ctDNA in blood samples. The sensitivity for CRC patients in their study was 83%. Analysis of other publications showed that the sensitivity of liquid biopsy for CRC patients ranged from 61% to 95%, with specificity ranging from 99.7% to 100% (Alizadeh-Sedigh et al., 2022; Nakamura et al., 2024).

In our study, liquid biopsy detected disease recurrence in radically resected patients 2.14 months earlier than CT and colonoscopy. The recurrence rate was 59.3% for ctDNA-positive patients and 9.9% for ctDNA-negative patients. Chen et al. (2021) identified ctDNA in CRC patients after radical surgery. Among 240 patients, 20 (8.3%) were ctDNA-positive. Of these, 12 (60%) showed radiologic progression an average of 5.01 months after ctDNA detection. 2-year RFS rate for ctDNA-negative patients was 89.4%, while for ctDNA-positive patients, it was 39.3%. Khakoo et al. (2020) and Suzuki et al. (2020) conducted similar studies using ctPCR on smaller groups of CRC patients. The average time from ctDNA detection to confirmed recurrence was 2.6 and 4.81 months, respectively.

Most studies focus on the prognostic value of ctDNA in metastatic CRC patients. Therefore, studies like ours remain relevant for accumulating data and creating new diagnostic systems. The limitations of this study were the relatively small sample size and short follow-up period (the median survival time was not reached).

Conclusions

The studied mutations were found in 34 (24.6%) out of 138 tumor tissue samples. The prevalence of mutations in the study cohort was: KRAS – 9.4%, BRAF – 8.7%, PIK3CA – 3.6%, TP53 – 4.4%. The presence of at least one of the studied mutations significantly affected RFS. The sensitivity of liquid biopsy for detecting ctDNA was 79.4%, and specificity was 100%. The recurrence rate was 59.3% for ctDNA-positive patients and 9.9% for ctDNA-negative patients. Liquid biopsy detected disease recurrence 2.14 months earlier than CT and colonoscopy.

Further studies with larger cohorts are needed for liquid biopsy standardization and validation of its clinical utility. Its implementation into routine monitoring could revolutionize early recurrence detection and therapeutic decision-making in oncology. Identification of high-risk patients before treatment initiation and ctDNA monitoring during therapy can help optimize treatment selection and reduce postoperative recurrence rates in CRC patients. Ultimately, our findings support the role of liquid biopsy, particularly ctDNA, in advancing personalized treatment strategies for CRC.

The authors declare no conflict of interest.

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