

# The effect of genetic alterations detected by the circulating tumor DNA-based next-generation sequencing technique on prognosis and survival in metastatic colorectal cancer

*Metastatik kolorektal kanserde dolaşan tümör DNA'sına dayalı yeni nesil dizileme tekniği ile tespit edilen genetik deęişikliklerin prognoz ve sağ kalım üzerine etkisi*

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## Abstract

**Purpose:** Studies conducted to date showed that circulating tumor DNA (ctDNA)-based next generation sequencing (NGS) panels are beneficial in the treatment strategies of patients with metastatic colorectal cancer (mCRC). In this study, we planned to determine the frequencies of various genetic alterations in patients with mCRC by ctDNA-based NGS analyses, evaluate the concordance rates by comparing these results with the results in standard polymerase chain reaction (PCR) analyses, and investigate the effect of the detected alterations on overall survival and progression-free survival.

**Materials and methods:** The study was conducted by retrospective screening and analysis of the data on 48 patients, who were followed up with a diagnosis of mCRC and who received chemotherapy and/or biological agents. The data were analyzed using SPSS 25.0 [IBM SPSS Statistics 25 software (Armonk, NY: IBM Corp.)] package program.

**Results:** In this study, ctDNA-based NGS analyses, compared to the quantitative PCR-based gold standard method, were found to have a sensitivity rate of 64.7%, specificity rate of 55.6% and concordance rate of 59.1% for KRAS mutation; a sensitivity rate of 100%, specificity rate of 86.7% and concordance rate of 87.1% for NRAS mutation; a sensitivity rate of 50%, specificity rate of 96.4% and concordance rate of 90.6% for BRAF mutation. In addition, concordance rates were evaluated based on the time elapsed between the time of taking the liquid biopsy and tissue biopsy samples. As a result, concordance rates for KRAS, NRAS, and BRAF mutations were found to be 60.9%, 100%, and 100% respectively, in cases where this elapsed time was less than 6 months; and were found to be 57.1%, 78.9%, and 85% respectively, in cases where this elapsed time was more than 6 months. Furthermore, the comprehensive analyzes revealed that the frequency of many molecular changes in mCRC as well as the relationship of these changes with clinicopathological features and survival times.

**Conclusion:** Our study demonstrates the clinical benefit of ctDNA-based NGS analyzes in patients with mCRC.

**Keywords:** Colorectal cancer, ctDNA, gene sequencing, liquid biopsy, next generation sequencing.

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## Öz

**Amaç:** Şu ana kadar yapılan çalışmalar; dolaşan tümör DNA'sı (ctDNA) tabanlı next-generation sequencing (NGS) panellerinin, metastatik kolorektal kanserli (mCRC) hastalarda tedavi stratejilerinde yarar sağladığını göstermiştir. Biz de bu çalışmamızda; mCRC'li hastalarda ctDNA tabanlı NGS analizleriyle çeşitli gen deęişikliklerinin sıklıklarını saptamayı, bu sonuçları standart polimeraz zincir reaksiyonu (PCR) analizlerindeki sonuçlarla karşılaştırarak uyum oranlarını deęerlendirmeyi ve saptanan deęişikliklerin genel sağ kalım ve progresyonsuz sağ kalıma etkisini araştırmayı planladık.

**Gereç ve yöntem:** Çalışma; mCRC tanısı ile takip edilen, kemoterapi ve/veya biyolojik ajan alan 48 hastaya ait bilgilerin retrospektif olarak taranması ve analiz edilmesi ile hazırlandı. Veriler SPSS 25.0 [IBM SPSS Statistics 25 software (Armonk, NY: IBM Corp.)] paket programı kullanılarak analiz edildi.

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**Bulgular:** Çalışmada; ctDNA tabanlı NGS analizlerinin, kantitatif PCR tabanlı altın standart yöntemle kıyasla KRAS mutasyonu için %64,7 duyarlılık, %55,6 özgüllük ve %59,1 uyum oranına; NRAS mutasyonu için %100 duyarlılık, %86,7 özgüllük ve %87,1 uyum oranına; BRAF mutasyonu için %50 duyarlılık, %96,4 özgüllük ve %90,6 uyum oranına sahip olduğu gösterildi.

Ayrıca, likit biyopsi ile doku biyopsisi örneklerinin alınma zamanları arasındaki süreye göre uyum oranları da değerlendirildi. Sonuçta; uyum oranları bu süre 6 aydan kısa olanlarda KRAS, NRAS, BRAF mutasyonları için sırasıyla %60,9, %100, %100 olurken; süre 6 aydan uzun olanlarda sırasıyla %57,1, %78,9, %85 olarak saptandı. Bunun yanında, yapılan kapsamlı analizler sonucunda; mCRC'de birçok moleküler değişikliğin sıklığı ve bu değişikliklerin klinikopatolojik özellikler ve sağ kalım süreleriyle ilişkisi ortaya koyuldu.

**Sonuç:** Çalışmamız; mCRC'li hastalarda, ctDNA tabanlı NGS analizlerinin klinik yararını göstermektedir.

**Anahtar kelimeler:** ctDNA, gen dizileme, kolorektal kanser, likit biyopsi, yeni nesil dizileme.

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## Introduction

With 1.9 million new cases each year, colorectal cancer is the 3rd most common and the 2nd most deadly type of cancer worldwide [1]. Approximately 20% of patients with colorectal cancer have a metastatic disease at the time of diagnosis, and the 5-year survival rate in these patients is 13% [2, 3]. Therefore, the focus in a significant part of cancer research has been on new diagnostic and therapeutic approaches for metastatic colorectal cancers (mCRC) in recent years [2, 4].

In recent years, many targetable molecular changes have been detected; especially after the next generation sequencing (NGS) technique's coming into use for cancer patients. This technique offers the advantages of preventing delays for patients and the ability to direct patients to the most appropriate clinical research by making it possible to sequence multiple genes at once instead of performing multiple sequential single tests [5].

NGS analyzes can be performed directly on samples taken from tumor tissue, as well as on materials taken by liquid biopsy from peripheral blood. This is because peripheral blood contains ctDNA (circulating tumor DNA) that offers a great opportunity for the use of detailed molecular techniques. Some studies have shown that mutations in ctDNA correspond exactly to mutations from the primary tumor. For this reason, it has been reported that ctDNA-based molecular analyses can be conducted to detect targetable molecular changes [6, 7]. The US Food and Drug Administration (FDA) has approved the use of ctDNA-based

Guardant360CDx and FoundationOne Liquid CDx tests in many types of cancer [8, 9]. Studies conducted to date showed that circulating tumor DNA (ctDNA)-based next generation sequencing (NGS) panels are beneficial in the treatment strategies of patients with mCRC [10].

Therefore, we planned in our study to determine the frequency of various gene changes in mCRC patients by conduction ctDNA-based NGS analyses; to evaluate concordance rates by comparing the results with the results of standard polymerase chain reaction (PCR) analyses; and to investigate the effect of the detected changes on overall survival and progression-free survival. With all this information, we aimed at contributing to the literature, in terms of identifying ideal personalized treatment procedures for mCRC patients and enhancing overall survival (OS) and progression-free survival (PFS).

## Materials and methods

### Study design and participants

The study was conducted by retrospective screening and analyzing the anamnesis, examination, laboratory and imaging data on 48 patients aged 18 years and over, who were being followed up with a diagnosis of mCRC and being treated with chemotherapy and/or a biological agent at Pamukkale University Faculty of Medicine, Medical Oncology Clinic. The study involved data from patients who were in the metastatic stage at the time of diagnosis or who developed metastases during follow-up. Patients whose medical records were not fully accessible, patients diagnosed with cancer in an

external center, and patients whose NGS and PCR analyzes were performed in an external center were not included in the study.

In our study; the results of quantitative PCR-based gold standard genomic DNA analyzes on tumor tissue samples were compared with the results of ctDNA-based NGS analyzes on peripheral blood samples. In our clinic, quantitative PCR-based genomic DNA analysis are conducted routinely only for the KRAS, NRAS, and BRAF genes in the group of patients with mCRC; and therefore, comparison was made based on the mutation rates in these genes. In addition, concordance rates of the results were evaluated based on the time elapsed between the time of taking the liquid biopsy and tissue biopsy samples (less than 6 months vs more than 6 months).

Demographic characteristics of the patients including age, gender, family history, smoking, and alcohol consumption were evaluated. The age variable is grouped as under 65 years of age, and 65 years of age and above. As family history, it was questioned whether there was a history of solid cancer in first-degree relatives. For the variable smoking, patients were classified into groups of those who had no history of smoking and those with a history of smoking (who were still an active smoker or who used to smoke but then quit). For the variable alcohol consumption, patients were classified into groups of those who had consumed alcohol, without specifying the amount, and those who had not.

The study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. The study protocol was approved by the Medical Ethics Committee of Pamukkale University Faculty of Medicine with No. 21 on 12.11.2020. Patients alive at the time of data collection provided informed consent.

### **Molecular analysis**

Samples taken from the patients were studied in the molecular laboratory of Pamukkale University, Department of Medical Genetics. 7 ml of peripheral blood samples taken from the patients were collected in pax gene tubes. Samples were then centrifuged for 20 minutes

at 1600 xg and for 20 minutes at 4000 xg, in order to separate the plasmas. Circulating free DNA (cfDNA) isolation from approximately 5 ml plasma samples was performed using Qiagen Qiamp Circulating Nucleic Acid Kit (kat:55114 Germany). Samples were measured in Nanodrop and were then involved in the study. From the obtained cfDNAs, a library was prepared using Accel-Amplicon 56 G Oncology Panel Kit for next-generation DNA sequencing. The prepared libraries were run on the Illumina MiSeq platform. The resulting Fastq files were analyzed on the Sophia DDM platform.

### **Statistical analysis**

The data were analyzed using SPSS 25.0 [IBM SPSS Statistics 25 software (Armonk, NY: IBM Corp.)] package program. Continuous variables were represented as mean  $\pm$  standard deviation (S.D.), while categorical variables were represented as frequencies and percentages. Pearson chi-square test was used in analyzing the relationships between categorical variables. Survival curves were calculated using Kaplan-Meier method, and were compared using the log-rank test.  $p < 0.05$  was considered to be statistically significant. Concordance analyses were performed using Cohen's kappa test.

## **Results**

### **Study population**

In the study, data belonging to a total of 48 patients diagnosed with mCRC, who met the inclusion criteria, were studied. Demographic and clinical characteristics of patients are shown in Table 1.

### **Results of NGS analysis**

In our study, we determined mutation frequencies in genes by conducting NGS analyses on ctDNA materials isolated from peripheral blood samples (Table 2).

The study also involved questioning whether there was a significant difference between the frequency of gene mutations, on the basis of the demographic and clinical characteristics of patients. Table 3 shows the groups with a statistically significant difference.

**Table 1.** Demographic and clinical characteristics (n=48)

Characteristics	n (%)	Characteristics	n (%)
<b>Age (year)</b>		<b>Metastasectomy</b>	
<65	34 (70.8)	Yes	9 (18.8)
≥65	14 (29.2)	No	39 (81.3)
<b>Gender</b>		<b>Radiofrequency ablation</b>	
Female	12 (25)	Yes	3 (6.3)
Male	36 (75)	No	45 (93.8)
<b>Family history of cancer</b>		<b>TAKE</b>	
Yes	11 (22.9)	Yes	8 (16.7)
No	37 (77.1)	No	40 (83.3)
<b>Smoking*</b>		<b>HIPEC</b>	
Yes	13 (37.1)	Yes	3 (6.3)
No	22 (62.9)	No	45 (93.8)
<b>Alcohol consumption**</b>		<b>Liver metastasis</b>	
Yes	3 (8.6)	Yes	26 (54.2)
No	32 (91.4)	No	22 (45.8)
<b>Tumor histology</b>		<b>Lung metastasis</b>	
Adenocarcinoma	38 (79.2)	Yes	23 (47.9)
Mucinous adenocarcinoma	10 (20.8)	No	25 (52.1)
<b>Primary tumor localization</b>		<b>Peritoneal metastasis</b>	
Right-sided colon	13 (27.1)	Yes	7 (14.6)
Left-sided colon	15 (31.3)	No	41 (85.4)
Rectum	20 (41.7)		
<b>Lymphovascular invasion</b>		<b>Bone metastasis</b>	
Yes	20 (64.5)	Yes	3 (6.3)
No	11 (35.5)	No	45 (93.8)
<b>Perineural invasion</b>		<b>CEA level</b>	
Yes	13 (43.3)	Normal	19 (40.4)
No	17 (56.7)	High (>4.7 ug/l)	28 (59.6)
<b>Microsatellite instability</b>		<b>CA 19-9 level</b>	
Yes	6 (26.1)	Normal	27 (57.4)
No	17 (73.9)	High (>27 u/ml)	20 (42.6)
<b>Resection of primary tumor</b>		<b>First-line regimen</b>	
Yes	35 (72.9)	Capecitabine	1 (2.6)
No	13 (27.1)	Xelox+bevacizumab	13 (34.2)
<b>Adjuvant therapy</b>		Xelox+cetuximab	1 (2.6)
Yes	25 (52.1)	Folfox+bevacizumab	3 (7.9)
No	23 (47.9)	Folfox+cetuximab	4 (10.5)
<b>Adjuvant therapy regimen</b>		Folfox+panitumumab	5 (13.2)
Xelox	20 (80)	Folfiri+Bevacizumab	3 (7.9)
Folfox	3 (12)	Folfiri+Cetuximab	4 (10.5)
De gramont	1 (4)	Folfiri+panitumumab	2 (5.3)
Capecitabine	1 (4)	Irinotecan+cetuximab	1 (2.6)
<b>Local therapy</b>		Oxaliplatin+panitumumab	1 (2.6)
Yes	29 (60.4)		
No	19 (39.6)		

**Table 1.** Demographic and clinical characteristics (n=48) (continued)

Characteristics	n (%)	Characteristics	n (%)
<b>Radiotherapy</b>		<b>First-line biological agent</b>	
Yes	14 (29.2)	Bevacizumab	19 (51.4)
No	34 (70.8)	Cetuximab	10 (27.0)
		Panitumumab	8 (21.6)

\*Smoking data could not be obtained for 13 patients, \*\*Alcohol consumption data could not be obtained for 13 patients

**Table 2.** Mutant gene rates obtained by NGS analysis (n>48)

Gene	n (%)	Gene	n (%)
ALK	0 (0)	GNAQ	1 (2.1)
ATM	8 (16.7)	GNAS	4 (8.3)
BRAF	4 (8.3)	JAK2	1 (2.1)
EGFR	37 (77.1)	JAK3	26 (54.2)
ERBB2	11 (22.9)	NOTCH1	34 (70.8)
HRAS	17 (35.4)	NPM1	0 (0)
IDH1	0 (0)	PIK3CA	37 (77.1)
IDH2	2 (4.2)	SMAD4	29 (60.4)
KDR	38 (79.2)	ABL1	16 (33.3)
KIT	18 (37.5)	AKT1	29 (60.4)
KRAS	26 (54.2)	CDH1	3 (6.3)
MAP2K1	20 (41.7)	CSF1R	32 (66.7)
MET	9 (18.8)	CTNNB1	1 (2.1)
NRAS	6 (12.5)	DDR2	0 (0)
PTEN	11 (22.9)	EZH2	6 (12.5)
RB1	25 (52.1)	FBXW7	25 (52.1)
RET	38 (79.2)	FOXL2	8 (16.7)
TP53	46 (95.8)	HNF1A	4 (8.3)
APC	27 (56.3)	MLH1	2 (4.2)
CDH1	5 (10.4)	MPL	4 (8.3)
CDKN2A	9 (18.8)	MSH6	8 (16.7)
DNMT3A	10 (20.8)	PDGFRA	16 (33.3)
ERBB4	29 (60.4)	SMARCB1	15 (31.3)
FGFR1	2 (4.2)	SMO	4 (8.3)
FGFR2	31 (64.6)	SRC	0 (0)
FGFR3	30 (62.5)	STK11	22 (45.8)
FLT3	18 (37.5)	TSCI	0 (0)
GNA11	17 (35.4)	VHL	35 (72.9)

**Table 3.** Groups with significant differences in gene mutation frequencies according to demographic and clinical characteristics

Mutation	Age				p value		
	<65		≥65				
	n	%	n	%			
KDR exon 30	2	5.9	6	42.9	0.005* X <sup>2</sup> =9.761		
Mutation	Smoking				p value		
	No		Yes				
	n	%	n	%			
EGFR exon 20	0	0	3	23.1	0.044* X <sup>2</sup> =5.553		
MET	1	4.5	5	38.5	0.019* X <sup>2</sup> =6.618		
Mutation	Family history of cancer				p value		
	No		Yes				
	n	%	n	%			
TP53	37	100	9	81.8	0.049* X <sup>2</sup> =7.020		
TP53 exon 5	12	32.4	0	0	0.044* X <sup>2</sup> =4.757		
ERBB4	26	70.3	3	27.3	0.016* X <sup>2</sup> =6.555		
ERBB4 exon 9	17	45.9	1	9.1	0.035* X <sup>2</sup> =4.914		
Mutation	Tumor histology				p value		
	Adenocarcinoma		Mucinous adenocarcinoma				
	n	%	n	%			
DNMT3A	5	13.2	5	50	0.022* X <sup>2</sup> =6.515		
TP53 exon 5	12	31.6	0	0	0.039* X <sup>2</sup> =4.211		
Mutation	Primary tumor localization						p value
	Right-sided colon		Left-sided colon		Rectum		
	n	%	n	%	n	%	
KRAS	5	38.5	12	80	9	45	0.050* X <sup>2</sup> =6.001
ERBB4	4	30.8	11	73.3	14	70	0.037* X <sup>2</sup> =6.593
RET exon 10	8	61.5	4	26.7	15	75	0.015* X <sup>2</sup> =8.339
Mutation	Lymphovascular invasion				p value		
	No		Yes				
	n	%	n	%			
CDKN2A	0	0	9	45	0.012* X <sup>2</sup> =6.975		
PIK3CA	4	36.4	16	80	0.023* X <sup>2</sup> =5.903		
PIK3CA exon 14	0	0	8	40	0.028* X <sup>2</sup> =5.930		
Mutation	Perineural invasion				p value		
	No		Yes				
	n	%	n	%			
MAP2K1	6	35.3	10	76.9	0.024* X <sup>2</sup> =5.129		
JAK3	6	35.3	11	84.6	0.007* X <sup>2</sup> =7.298		
SMO	0	0	4	30.8	0.026* X <sup>2</sup> =6.036		
Mutation	Microsatellite instability				p value		
	No		Yes				
	n	%	n	%			
KDR	16	94.1	3	50	0.040* X <sup>2</sup> =6.008		
PDGFRA	3	17.6	5	83.3	0.009* X <sup>2</sup> =8.435		
EGFR exon 21	3	17.6	4	66.7	0.045* X <sup>2</sup> =5.033		

**Table 3.** Groups with significant differences in gene mutation frequencies according to demographic and clinical characteristics (continued)

<b>Liver metastasis</b>					
<b>Mutation</b>	<b>No</b>		<b>Yes</b>		<b>p value</b>
	<b>n</b>	<b>%</b>	<b>n</b>	<b>%</b>	
EGFR	20	90.9	17	65.4	0.036* X <sup>2</sup> =4.395
EGFR exon 7	13	59.1	5	19.2	0.004* X <sup>2</sup> =8.078
KDR exon 11	7	31.8	16	61.5	0.040* X <sup>2</sup> =4.218
ERBB4 exon 9	13	59.1	5	19.2	0.004* X <sup>2</sup> =8.078
FBXW7	18	81.8	7	26.9	0.000* X <sup>2</sup> =14.389
VHL exon 2	11	50	5	19.2	0.024* X <sup>2</sup> =5.077
<b>Lung metastasis</b>					
<b>Mutation</b>	<b>No</b>		<b>Yes</b>		<b>p value</b>
	<b>n</b>	<b>%</b>	<b>n</b>	<b>%</b>	
MAP2K1	14	56	6	26.1	0.036* X <sup>2</sup> =4.410
MAP2K1 exon 6	8	32	1	4.3	0.024* X <sup>2</sup> =6.013
PTEN exon 2	0	0	4	17.4	0.046* X <sup>2</sup> =4.743
TP53 exon 5	3	12	9	39.1	0.030* X <sup>2</sup> =4.703
ERBB4 exon 9	6	24	12	52.2	0.044* X <sup>2</sup> =4.057
FGFR3	11	44	19	82.6	0.006* X <sup>2</sup> =7.619
FGFR3 exon 9	3	12	9	39.1	0.030* X <sup>2</sup> =4.703
FBXW7	9	36	16	69.6	0.020* X <sup>2</sup> =5.408
FBXW7 exon 9	8	32	14	60.9	0.045* X <sup>2</sup> =4.022
<b>Peritoneal metastasis</b>					
<b>Mutation</b>	<b>No</b>		<b>Yes</b>		<b>p value</b>
	<b>N</b>	<b>%</b>	<b>n</b>	<b>%</b>	
KDR exon 11	23	56.1	0	0	0.010* X <sup>2</sup> =7.540
<b>Bone metastasis</b>					
<b>Mutation</b>	<b>No</b>		<b>Yes</b>		<b>p value</b>
	<b>n</b>	<b>%</b>	<b>n</b>	<b>%</b>	
KRAS exon 2	6	13.3	3	100	0.005* X <sup>2</sup> =13.867
PTEN exon 8	1	2.2	2	66.7	0.008* X <sup>2</sup> =19.935
APC exon 16	15	33.3	3	100	0.047* X <sup>2</sup> =5.333
VHL	35	77.8	0	0	0.017* X <sup>2</sup> =8.615
<b>CEA level</b>					
<b>Mutation</b>	<b>Normal</b>		<b>High</b>		<b>p value</b>
	<b>n</b>	<b>%</b>	<b>n</b>	<b>%</b>	
KRAS	6	31.6	19	67.9	0.014* X <sup>2</sup> =5.983
RB1	14	73.7	11	39.3	0.020* X <sup>2</sup> =5.379
<b>CA 19-9 level</b>					
<b>Mutation</b>	<b>Normal</b>		<b>High</b>		<b>p value</b>
	<b>n</b>	<b>%</b>	<b>n</b>	<b>%</b>	
FLT3 exon 11	0	0	4	20	0.027* X <sup>2</sup> =5.902

\*Pearson chi-square test

### Liquid biopsy-tissue biopsy concordance analysis

In our study; the results of quantitative PCR-based gold standard genomic DNA analyzes on tumor tissue samples were compared with the results of ctDNA-based NGS analyzes on peripheral blood samples. In our clinic, quantitative PCR-based genomic DNA analysis are conducted routinely only for the KRAS, NRAS, and BRAF genes in the group of patients with mCRC; and therefore, comparison was made based on the mutation rates in these genes. During the investigation of the KRAS gene, 11 of the 17 patients, who were found to have a KRAS mutation in their PCR analyzes, were also found to have mutations in their NGS analyzes, while no mutations were detected in NGS analyzes of 6 among them (64.7% sensitivity). Of the 27 patients who were not found to have a KRAS mutation in their PCR analyzes, 15 patients were found to have no mutation while 12 were found to have KRAS gene mutation in their NGS analyzes (55.6% specificity). In general, NGS analyzes performed for the KRAS gene showed a result compatible with the PCR analyzes in 26 of 44 patients (59.1% concordance rate). During the investigation of the NRAS gene, the PCR analysis detected NRAS mutation in only 1 patient, who was also found to have NRAS mutation in his NGS analysis (100% sensitivity). Of the 30 patients who were not found to have a NRAS mutation in their PCR analyzes, 26 patients were found to have no mutation while 4 were found to have NRAS gene mutation in their NGS analyzes (86.7% specificity). In general, NGS analyzes performed for the NRAS gene showed a result compatible with the PCR analyzes in 27 of 31 patients (87.1% concordance rate). During the investigation of the BRAF gene, 2 of the 4 patients, who were found to have a BRAF mutation in their PCR analyzes, were also found to have mutations in their NGS analyzes, while no mutations were detected in NGS analyzes of 2 among them (50% sensitivity). Of the 28 patients who were not found to have a BRAF mutation in their PCR analyzes, 27 patients were found to have no mutation while 1 were found to have BRAF gene mutation in their NGS analyzes

(96.4% specificity). In general, NGS analyzes performed for the BRAF gene showed a result compatible with the PCR analyzes in 29 of 32 patients (90.6% concordance rate) (Table 4).

In addition, concordance rates of the results were evaluated based on the time elapsed between the time of taking the liquid biopsy and tissue biopsy samples. During the investigation of KRAS mutation, NGS analysis gave results compatible with PCR analysis in 14 (60.9%) of 23 cases where this elapsed time was less than 6 months, while results compatible with PCR analysis in 12 (57.1%) of 21 cases, where this elapsed time was more than 6 months. During the investigation of NRAS mutation, NGS analysis gave results compatible with PCR analysis in 12 (100%) of 12 cases where this elapsed time was less than 6 months, while results compatible with PCR analysis in 15 (78.9%) of 19 cases, where this elapsed time was more than 6 months. During the investigation of BRAF mutation, NGS analysis gave results compatible with PCR analysis in 15 (100%) of 15 cases where this elapsed time was less than 6 months, while results compatible with PCR analysis in 17 (85%) of 20 cases, where this elapsed time was more than 6 months. As a result, the concordance rate for cases, in which this elapsed time was less than 6 months, was 60.9% for KRAS, 100% for NRAS, and 100% for BRAF, while for cases, in which this elapsed time was more than 6 months, the concordance rate was 57.1% for KRAS, 78.9% for NRAS, and 85% for BRAF (Table 5).

### Survival analysis

In our study, the mean overall survival time of the all patient group was calculated to be 59.3 months ( $\pm 11.0$  95% confidence interval [CI]: 37.7-80.9), while the progression-free survival time was calculated to be 18.8 months ( $\pm 3.5$  95% CI: 11.9-25.7). In the study, analyses on overall survival and progression-free survival were conducted according to the mutation status in the genes, and the groups found to have statistically significant differences were shown in Table 6 and Table 7. Survival curves were shown in Figure 1 and Figure 2.



**Table 4.** Liquid biopsy-tissue biopsy concordance analysis

Gene	Concordance	Sensitivity	Specificity	Kappa	p value
<b>KRAS</b>	59.1%	64.7%	55.6%	0.190	0.190
<b>NRAS</b>	87.1%	100%	86.7%	0.295	0.020*
<b>BRAF</b>	90.6%	50%	96.4%	0.520	0.003*

\*Cohen's kappa test

**Table 5.** Concordance rates of analysis results according to the time between collection of liquid biopsy and tissue biopsy samples

Gene	Time interval (months)	Concordance	Kappa	p value
<b>KRAS</b>	<6	60.9%	0.219	0.292
	>6	57.1%	0.160	0.407
<b>NRAS</b>	<6	100%	1.000	0.001*
	>6	78.9%	-	-
<b>BRAF</b>	<6	100%	1.000	0.001*
	>6	85%	0.318	0.144

\*Cohen's kappa test

**Table 6.** Groups with significant differences in overall survival according to mutation status

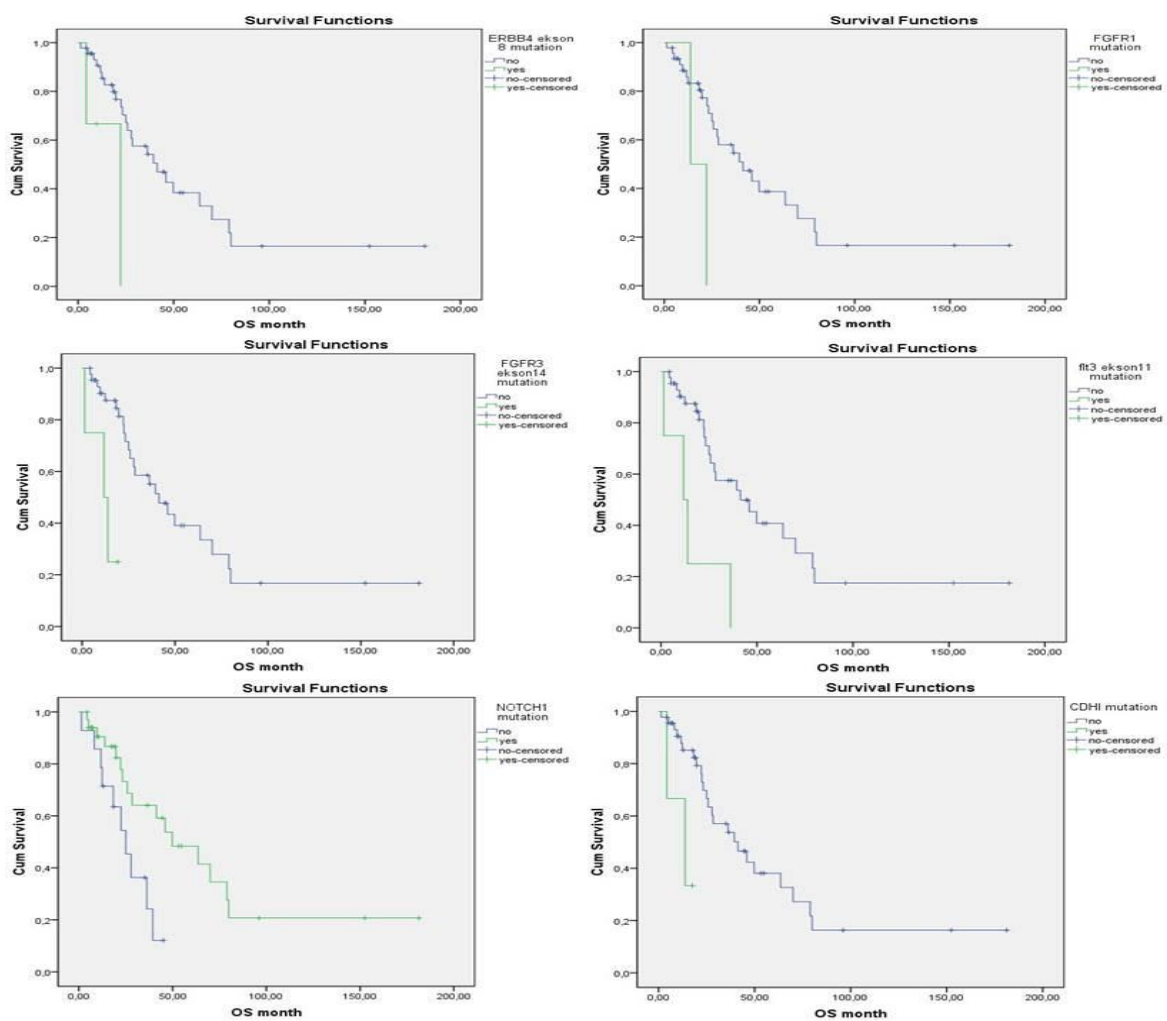
Gene	Mutation	Mean	S. D.	95% CI	p value
<b>ERBB4 exon8</b>	No	61.714	11.475	39.224-84.204	0.022*
	Yes	16.233	6.890	2.729-29.737	
<b>FGFR1</b>	No	61.882	11.537	39.269-84.496	0.034*
	Yes	18.017	4.183	9.817-26.216	
<b>FGFR3 exon 14</b>	No	62.941	11.619	40.166-85.715	0.001*
	Yes	11.542	3.240	5.191-17.892	
<b>FLT3 exon 11</b>	No	64.297	12.018	40.742-87.852	0.002*
	Yes	15.800	7.346	1.401-30.199	
<b>NOTCH1</b>	No	25.290	3.711	18.017-32.562	0.012*
	Yes	71.545	14.016	44.075-99.016	
<b>CDH1</b>	No	61.581	11.407	39.223-83.938	0.017*
	Yes	11.889	3.219	5.581-18.197	
<b>CSF1R exon 22</b>	No	62.890	11.768	39.824-85.956	0.013*
	Yes	16.456	10.348	0.000-36.737	
<b>MSH6</b>	No	66.624	12.715	41.702-91.547	0.011*
	Yes	22.610	6.062	10.729-34.491	
<b>SMARCB1 exon 5</b>	No	67.046	12.963	41.638-92.454	0.032*
	Yes	25.045	4.990	15.264-34.826	
<b>STK11</b>	No	38.268	9.354	19.933-56.602	0.009*
	Yes	80.539	16.472	48.254-112.825	
<b>PIK3CA exon 10</b>	No	65.711	12.327	41.550-89.872	0.005*
	Yes	18.817	3.393	12.166-25.468	

\* log-rank test

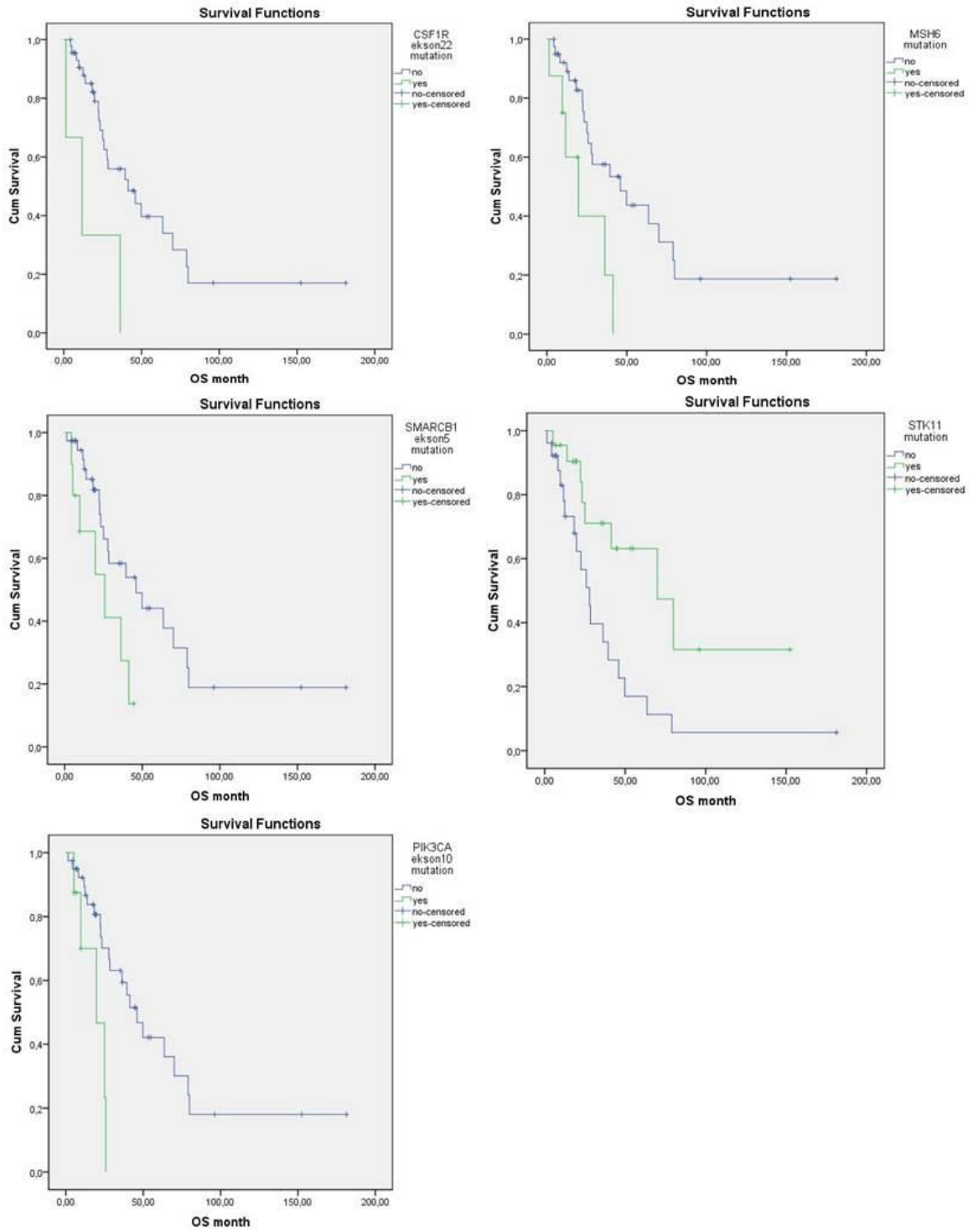
**Table 7.** Groups with significant differences in progression-free survival according to mutation status

Gene	Mutation	Mean	S. D.	95% CI	p value
EGFR exon 20	No	19.693	3.676	12.488-26.898	0.014*
	Yes	5.367	4.833	0.000-14.840	
KIT exon 10	No	15.887	2.770	10.459-21.316	0.034*
	Yes	22.320	2.159	18.089-26.551	
PTEN exon 8	No	19.747	3.671	12.553-26.942	0.002*
	Yes	5.956	3.131	0.000-12.091	
JAK3	No	11.815	1.941	8.010-15.621	0.017*
	Yes	26.340	5.402	15.751-36.929	
PIK3CA exon 10	No	20.044	3.763	12.667-27.420	0.002*
	Yes	6.993	1.147	4.745-9.242	
FBXW7	No	13.030	1.704	9.689-16.370	0.042*
	Yes	25.710	6.924	12.138-39.282	
STK11	No	10.907	1.261	8.436-13.378	0.001*
	Yes	27.207	6.252	14.954-39.460	

\* log-rank test



**Figure 1.** Survival curves of groups with significant differences in overall survival according to mutation status



**Figure 2.** Survival curves of groups with significant differences in progression-free survival according to mutation status

## Discussion

Mutations is detected in the KRAS gene in 35-45% of all CRC cases [11]. In a study; disease-free survival times were observed to be shorter in patients with KRAS exon 2 mutation than in patients without this mutation [12]. In our study, the frequency of mutations in the KRAS gene was found to be 54.2%. However, no difference statistically significant in terms of OS and PFS was observed according to the KRAS mutation status. As for the NRAS gene, mutations are detected in 5% of all CRC cases [13]. In the studies, it has been determined that the overall survival time of patients with NRAS mutations is significantly lower than that of patients with RAS wild type [14]. In our study, the NRAS gene was detected as a mutant gene in 12.5% of patients. However, no difference statistically significant in terms of OS and PFS was observed according to the NRAS mutation status.

Mutations in the BRAF gene are detected in approximately 8-12% of mCRC cases [15]. In a CRYSTAL study, BRAF mutation in mCRC was shown to be associated with a poor prognosis [16]. Consistent with the literature, the BRAF gene was found to be mutant in 8.3% of patients in our study. However, no difference statistically significant in terms of OS and PFS was observed according to the BRAF mutation status.

The PIK3CA gene is found to be mutated in about 80% of CRC cases [13]. Some studies have provided evidence that PIK3CA mutation is associated with resistance to anti-EGFR therapy [17]. Consistent with the literature, the PIK3CA gene was found to be mutant in 77.1% of patients in our study. In addition, the presence of PIK3CA exon 10 mutation was found to be associated with statistically significantly worse overall survival and progression-free survival times.

In the TCGA (Tumor Cancer Genome Atlas) dataset published in 2012, findings were obtained that suggest that mutations in the ERBB4 gene create a survival disadvantage in CRC [18]. In our study, the frequency of ERBB4 gene mutation was calculated to be 60.4%. In addition, the presence of ERBB4 exon 8 mutation was found to be associated with statistically significantly worse overall survival times.

Mutations in FBXW7, EGFR, JAK3, KIT, CSF1R, CDHI, FLT3, FGFR1, FGFR3, SMARCB1, PTEN, MSH6, NOTCH1, STK11 genes, which are among the genes analyzed in this study, have not clearly known roles in the pathogenesis and prognosis of CRC. Our study showed that mutations in the CDHI, MSH6, FGFR1, FGFR3 exon 14, CSF1R exon 22, FLT3 exon 11, and SMARCB1 exon 5 genes were associated with statistically significantly worse overall survival times; mutations in the NOTCH1 and STK11 genes were associated with better overall survival times; mutations in the EGFR exon 20 and PTEN genes were associated with worse progression-free survival times; and mutations in the STK11, FBXW7, JAK3, and KIT exon 10 genes were associated with better progression-free survival times.

In a study published in 2014, tissue samples taken from 106 patients were analyzed using the quantitative PCR-based gold standard method, while blood samples were analyzed using the ctDNA-based NGS technique, and the analysis of ctDNA showed 98% specificity, 92% sensitivity, and 96% concordance rates for KRAS mutation [19]. In a similar study conducted in 2018 showed 67% sensitivity, 90% specificity and 81% concordance rates for KRAS mutation [20]. In a study published in *Cancer Medicine* in 2019, which enrolled 101 patients with mCRC, the overall concordance rate between ctDNA and tissue analyzes was calculated to be 77.2%, in terms of the RAS mutation status [21]. In our study, ctDNA-based NGS analyses conducted on peripheral blood samples, compared to the quantitative PCR-based gold standard method used with tissue samples, were found to have a sensitivity rate of 64.7%, specificity of 55.6% and concordance rate of 59.1% for KRAS mutation; a sensitivity rate of 100%, specificity of 86.7% and concordance rate of 87.1% for NRAS mutation; and a sensitivity rate of 50%, specificity of 96.4% and concordance rate of 90.6% for BRAF mutation. As a result, it was revealed that ctDNA-based NGS analyses can be a good option for detecting molecular changes in patients with mCRC.

The findings of the limited number of studies in the literature suggested that the time elapsed between the sampling times of the liquid biopsy and tissue biopsy procedures has an effect on the concordance between the results of the

molecular analyzes conducted on the samples. In a 2020 study that enrolled 54 patients with mCRC, the rate of concordance between the results of the liquid biopsy and tissue biopsy procedures was found to be 50% in cases where the time elapsed between the sampling times of these procedures was more than 6 months and 83.1% in cases where this elapsed time was less than 6 months [22]. In another study that compared the results of the ctDNA analyses performed on samples taken from peripheral blood of 101 patients and the results of the genomic DNA analyses on samples taken from tumor tissues of these patients, the concordance rates were found to be 63% for TP53, 69% for EGFR, 85% for PIK3CA, and 87% for ERBB2 in cases where the time elapsed between the sampling times of the procedures was more than 6 months; while the concordance rates were found to be 82.1% for TP53, 71% for EGFR, 90% for PIK3CA, and 97% for ERBB2 in cases where this elapsed time was less than 6 months [23]. In the light of these data, in our study, concordance of the rates were also evaluated based on the time elapsed between the time of taking the liquid biopsy and tissue biopsy samples. The concordance rate for cases, in which this elapsed time was less than 6 months, was 60.9% for KRAS, 100% for NRAS, and 100% for BRAF, while for cases, in which this elapsed time was more than 6 months, the concordance rate was 57.1% for KRAS, 78.9% for NRAS, and 85% for BRAF. As a result, it was revealed that the time elapsed between the sampling times of the liquid biopsy and tissue biopsy procedures affects the concordance between the results of molecular analyzes of the samples and that ensuring this elapsed time to be shorter would contribute to the ability to achieve better results. Limitations of our study: the number of patients is relatively small, and the study is retrospective and single-center.

In conclusion, our study is one of the studies in the literature, which show the clinical benefit of ctDNA-based NGS analyzes for mCRC patients. Our study showed that ctDNA-based NGS analyzes gives results highly consistent with the results of quantitative PCR-based gold standard genomic DNA analyzes conducted on tumor tissue samples; and that this concordance is much higher in cases where the time elapsed between the sampling times of these

procedures was less than 6 months. In addition, the comprehensive analyzes revealed that the frequency of many molecular changes in mCRC as well as the relationship of these changes with clinicopathological features and survival times.

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**Authors contributions:** A.U.; Collected and analysed data, searched literature, wrote the manuscript.

A.G.D.; Designed study, agreed to be accountable for all aspects of the work.

A.D.; Analysed data.

A.Y.; Searched literature, collected data.

H.A.; Developed the theoretical framework.

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