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THE MUTATION PROFILES OF K-RAS/N-RAS GENES IN METASTATIC COLORECTAL CANCER PATIENTS

METASTATİK KOLOREKTAL KANSERLİ HASTALARDA KRAS/NRAS GEN MUTASYON PROFİLLERİ

Seda Eren Keskin[1*], Eda Güzdolu[2], Nilüfer Sertdemir[2], Gülhan Demir[2], Deniz Sünnetçi[1], Devrim Çabuk[3], Naci Çine[1], Hakan Savlı[1]

[1]Kocaeli University, Faculty of Medicine, Department of Medical Genetics, Kocaeli, Turkey, [2]Kocaeli University, Institute of Health Sciences, Department of Medical Genetics and Molecular Biology, Kocaeli, Turkey, [3]Kocaeli University, Faculty of Medicine, Department of Medical Oncology, Kocaeli, Turkey

ORCID iD: Seda Eren Keskin: 0000-0002-8315-646X; Eda Güzdolu: 0000-0001-6402-8828; Nilüfer Sertdemir: 000-0002-4138-7454; Gülhan Demir: 0000-0002-5110-2862; Deniz Sünnetçi: 0000-0001-9297-8222; Devrim Çabuk: 0000-0002-7871-6311; Naci Çine: 0000-0001-9063-1073; Hakan Savlı: 0000-0003-2836-9881

***Sorumlu Yazar / Corresponding Author:** Seda Eren Keskin, **e-posta / e-mail:** seda.eren@kocaeli.edu.tr

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Abstract

Objective: *RAS* genes are members of the *RAS*/Mitogen-activated protein kinase pathway which is induced by Epidermal Growth Factor Receptor (EGFR). Mutations in genes in this pathway trigger cancer development. In colorectal cancer, mutations in *RAS* genes cause resistance to EGRF-targeted therapy. In the treatment of metastatic colorectal cancer, EGFR's monoclonal antibodies are widely used as chemotherapeutic agents. Kirsten-*RAS* mutations are found in 30-50% and *N-RAS* mutations are found in 2-3% of colorectal cancer. In this study, we aimed to analyze Kirsten-*RAS* /*N-RAS* mutations in patients with metastatic colorectal cancer.
Methods: One hundred of metastatic colorectal cancer patients resistant to EGFR- targeted therapy were scanned for the Kirsten-*RAS* mutations status (exon 2,3,4) and *N-RAS* mutation status (Exon 2,3,4) by Real-Time PCR (Polymerase Chain Reaction) method.
Results: As a result of this study, *Kirsten-RAS* mutation was found 48% and *N-RAS* mutation was 1.92%. The most common *Kirsten-RAS* mutations were in codon 12. The distribution of codon 12 mutations were obtained as G12V (25%), G12D (23%), G12C (14.5%).
Conclusion: In our study, the frequencies of *Kirsten-RAS* and *N-RAS* mutations were compitable with similar reports. Our results have supported that testing RAS genes mutations have a vital role in identifying patients who benefit from Epidermal Growth Factor Receptor- targeted therapy.

Keywords: *EGFR-targeted therapy, KRAS, Metastatic colorectal cancer, NRAS, RAS oncogene*

Öz

Amaç: *RAS* genleri, Epidermal Büyüme Faktörü Reseptörü (*EGFR*) tarafından indüklenen *RAS-MAPK* Sinyal yolağının bir üyesidir. Bu yolaktaki genlerde meydana gelen mutasyonlar kanser gelişimini tetiklemektedir. Kolorektal kanserde (KRK), *RAS* genlerinde meydana gelen mutasyonlar EGFR hedefli tedaviye karşı direnç gelişimine neden olur. *EGFR* monoklonal antikorları, kemoterapötik ajanlar olarak metastatik kolorektal kanser tedavisinde yaygın şekilde kullanılmaktadır. *KRAS* mutasyonları KRK'nın 30-50%'sinde, *NRAS* mutasyonları ise 2-3%'ünde bulunur. Bu çalışmada, KRK'lı hastalarda *KRAS/NRAS* mutasyonlarını analiz etmeyi amaçladık.
Yöntem: EGFR-hedefli tedaviye direnç gösteren 100 metastatik KRK hastası, Real-Time Polimeraz Zincir Reaksiyonu yöntemi ile *KRAS* mutasyonu (ekzon 2, 3, 4) ve *NRAS* mutasyonu (ekzon 2, 3, 4) durumu için tarandı.
Bulgular: Bu çalışma sonucunda, *KRAS* mutasyonu oranı 48% ve *NRAS* mutasyonu oranı 1,92% olarak bulundu. En yaygın *KRAS* mutasyonları kodon 12'de saptandı. Kodon 12 mutasyonlarının dağılımı G12V (25%), G12D (23%), G12C (14,5%) olarak elde edildi.
Sonuç: Çalışmamızda saptanan *KRAS* ve *NRAS* mutasyon sıklıkları benzer raporlar ile uyumlu bulundu. Sonuçlarımız, *RAS* mutasyonlarının test edilmesinin *EGFR*-hedefli tedaviden fayda sağlayacak hastaları belirlemede hayati rolünü desteklemektedir.

Anahtar Kelimeler: *EGFR-hedefli Tedavi, KRAS, Metastatik Kolorektal Kanser, NRAS, RAS Onkogeni*

Introduction

Colorectal cancer (CRC) is accumulation of genetic and epigenetic differentiations which normal colon epithelium changes to invasive cancer cells. Mutations in genes in signaling pathways that regulate cell, growth, differentiation and apoptosis provoke the development of CRC.[1]

Epidermal Growth Factor Receptor (EGFR) and activates intracellular signaling pathways such as Rat sarcoma viral oncogene homolog (*RAS*)/Mitogen-activated protein kinase (*MAPK*). *RAS* genes are members of the *RAS-MAPK* pathway which are GTP-binding proto-oncogenes. *RAS* mutations cause the loss of GTPase activity of the RAS proteins. Abnormal forms of *RAS*-GTP results in cancer development by causing uncontrolled cell division.[2,3]

Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutations are reported as 30-50% and NRAS mutations are reported as 1-7% of CRC. *KRAS* mutations occur early stage of CRC and cause to increase the size of adenomatous tumour lesions. Somatic mutations in tumours occur at codons 12, 13, 61 or 146. In CRC, mutations of *KRAS* in codon 12 is the most common mutation.[4]

Surgery, chemotherapy and targeted therapies are used in the treatment of colorectal cancer. Targeted therapies are an effective alternative for patients have unresectable tumors. Anti-EGFR therapy is one of the leading targeted-therapy protocols in metastatic colorectal cancer (mCRC) treatment.[5] The status of KRAS mutations affect anti-EGFR therapy response by causing resistance. KRAS mutations have been tested routinely by PCR or sequencing based methods before mCRC treatment.[6]

This study aimed to present the frequencies and distributions of *KRAS* mutations in patients with CRC in our region by determining the *KRAS* mutation profiles.

Methods

We analyzed mutations of *KRAS-NRAS* genes in 100 patients with mCRC with Real-Time PCR method**.** Ethics committee approval KÜ GOKAEK 2017/3.7⁻2017.03.01_2017/32 and written consent from all participants were obtained.

Patient Group

One hundred patients resistant to EGFR targeted therapy with CRC between 2014-2018 years were included in our study. Formalin-fixed paraffin-embedded (FFPE) tissue sections were retrieved from Pathology Department. Clinical and demographic data (age, sex, tumor location, metastatic site) was obtained from pathology reports and medical records. Our study group consisted of 67 male and 33 female patients with ages 27 to 80. All patients had various metastases. The most common distant metastasis was liver metastasis. The ratio of metastasis in liver, lung, lymph node, brain, bone were in 61 (61%), 32 (32%), 32 (32%), 1 (1%), 4 (4%) patients, respectively (Table 1).

DNA Extraction

Genomic DNA was extracted from FFPE tissue sections according to the AmoyDx FFPE DNA Kit (Amoy Diagnostics Co., Ltd., China) protocol. The concentration of extracted DNA and the OD260/OD280 value was measured with spectrophotometer.

Mutation Analysis

First, mutation detection tests were performed according to the AmoyDx *KRAS* Mutation Detection Kit (Amoy Diagnostics Co., Ltd., China) protocol with Real-Time PCR method (Lightcycler 480 II, Roche Diagnostics).

The AmoyDx® KRAS Mutation Detection Kit is able to detect 19 somatic mutations in codons 12, 13, 59, 61, 117 and 146 of KRAS gene in human genomic DNA extracted from FFPE tumor sections.

As the second step, *NRAS* detection tests were for *KRAS* wild type patients. *NRAS* detection tests were according to the AmoyDx *NRAS* Mutation Detection Kit (Amoy Diagnostics Co., Ltd., China) protocol on LightCycler 480 II Real-Time PCR. All scanned mutations were listed in Table 2.

The AmoyDx NRAS Mutation Detection Kit is able to detect 16 somatic mutations in codons 12, 13, 59, 61, 117 and 146 of NRAS. According to manufacturer's protocol, master mixes wer prepared for each mutation seperately, and dispensed the mixes on the plate with patient DNAs and control DNA. Data Analysis were performed with LightCycler 480 II Real-Time PCR software.

Statistical Analysis

Basic statistics calculations were done using Microsoft Excel 2013. Differences in mutation frequencies between gender, age and metastases were tested for significance by t-test. ($p<0,05$ was considered as significant).

Results

We detected mutations in 48 (n=100, 48%) patients on exon 2, 3, 4 of the *KRAS* and 1 patients (n=52, 1,92%) on exon 2, 3, 4 of the *NRAS* gene (Table 3). Mutations in *KRAS* codon 12, 13, 61, 146 were detected in 36, 2, 8, 2 patients, respectively. Mutations were dominantly detected in codon 12. Detailed *KRAS* mutation patterns were shown in Figure 1. Mutant *NRAS* genotype was detected in only one (1.92%) of wild type *KRAS* patients. Fifty-two patients (52%) had tumors with no mutation. Two patients had multiple mutations. One patient was carrying G12C-G12R and the other carrying G12D-G12C mutations together. No *KRAS* G13C and A59T/Q61K mutations were detected.

Among *KRAS* mutation positive cases, there were 33 males (69%), 15 females (31%). The distribution of the *KRAS* mutation patterns according to gender was shown in Figure 2. The highest number of mutant *KRAS* cases was seen in the 60–69 years age group. No mutation was observed between the ages of 20-39 years. The distribution of the *KRAS* mutation patterns according to age groups was presented in Figure 3.

Table 1. Characteristics of patients.

Characteristics	n (%)	Mutation of KRAS (%)
Gender		
Male	67 (67%)	49.25%
Female	33 (33%)	45.45%
Age		
Range	27-80	48.00%
≤70	86 (86%)	43.02%
≥71	14 (14%)	78.57%
Tumor Location		
Rectum	23 (23.8%)	50.00%
Colon	76 (76.2%)	47.60%
Metastasis		
Liver	61 (61%)	46.70%
Lung	32 (32%)	69.70%
Lymph Node	32 (32%)	60.00%

Table 2. Mutations scanned by AmoyDx KRAS/NRAS kit

KRAS			
Exon	**Codon**	**Amino acid exchanges**	**Base changes**
Exon 2	Codon 12	Gly12Asp	35G>A
		Gly12Ala	35G>C
		Gly12Val	35G>T
		Gly12Ser	34G>A
		Gly12Arg	35G>C
		Gly12Cys	35G>T
	Codon 13	Gly13Asp	38G>A
		Gly13Cys	37G>T
Exon 3	Codon 59	Ala59Thr	175G>A
	Codon 61	Gln61Lys	181C>A
		Gln61Leu	182A>T
		Gln61Arg	182A>G
		Gln61His	183A>C
		Gln61His	183A>T
Exon 4	Codon 117	Lys117Asn	351A>C
		Lys117Asn	351A>T
	Codon 146	Ala146Thr	436G>A
		Ala146Val	437C>T
		Ala146Pro	436G>C
NRAS			
Exon	**Codon**	**Amino acid exchanges**	**Base changes**
Exon 2	Codon 12	Gly12Asp	35G>A
		Gly12Ser	34G>A
		Gly12Cys	34G>T
		Gly12Val	35G>T
		Gly12Ala	35G>C
	Codon 13	Gly13Asp	38G>A
		Gly13Arg	37G>C
		Gly13Val	38G>T
Exon 3	Codon 59	Ala59Asp	176C>A
	Codon 61	Gln61Arg	182A>G
		Gln61Lys	181C>A
		Gln61Leu	182A>T
		Gln61His	183A>C
Exon 4	Codon 117	Lys117Asn	351G>C
		Lys117Asn	351G>T
	Codon 146	Ala146Thr	436G>A

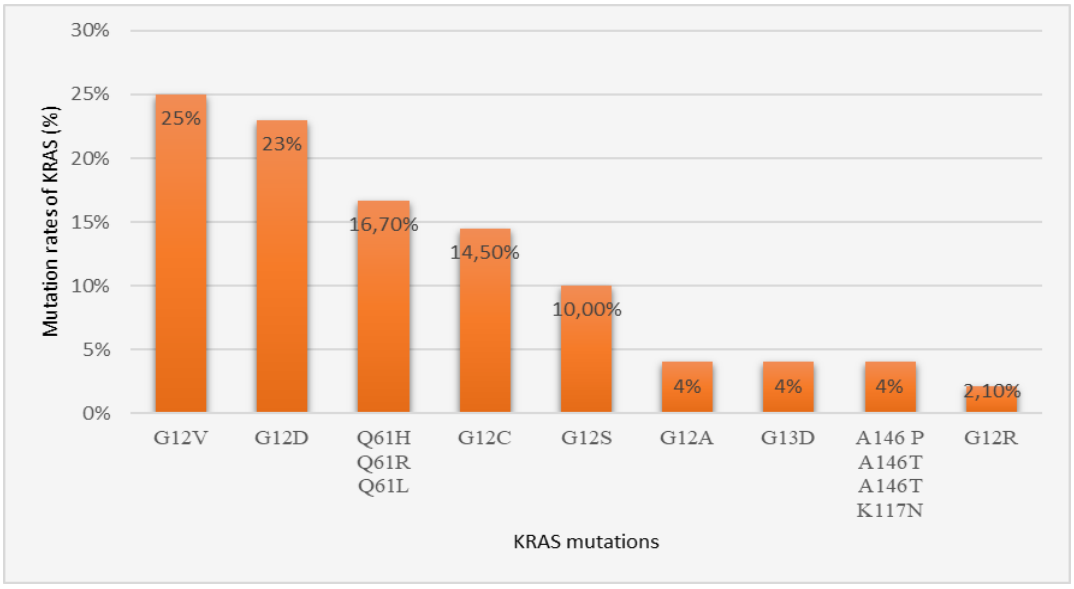

Figure 1. Patterns of KRAS mutations

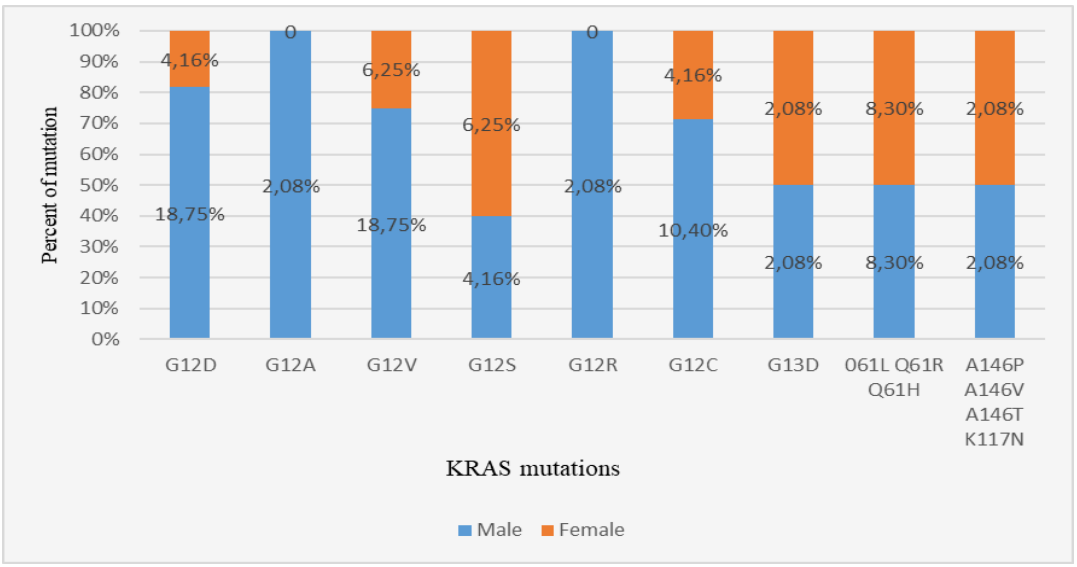

Figure 2. Frequencies of KRAS mutations according to gender.

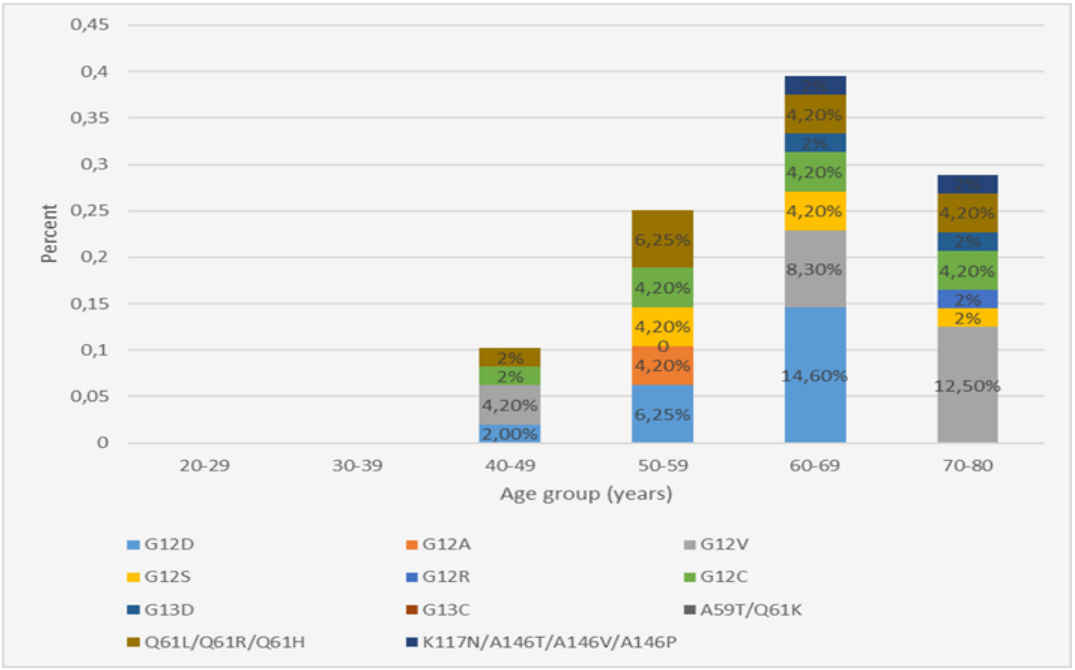

Figure 3. Frequencies of KRAS mutations according to age groups.

Discussion

Several genetic and epigenetic alterations, such as abnormal DNA mismatch repair and mutation of *KRAS, NRAS, BRAF, PIK3CA* participate in tumorigenesis and tumor progression of CRC. These alterations tend to affect the prognosis negatively.[7] In this study, we elucidated the prevalence of *KRAS* and *NRAS* mutations in 100 mCRC patients.

KRAS mutations occur in 30-50% of mCRC[8], in our study group, the frequency of *KRAS* gene mutations was 48%. Based on Table 3, The frequency of *KRAS* mutation in our study was determined similar to some studies that scanned the same codons from USA, Saudi Arabia, Lebanon, Japan and Turkey.[9,10,11,12] The frequency of *KRAS* mutation in our study was determined higher than countries such as India, Northeastern Iran and Turkey.[13,14,15] These differences between frequencies may be due to population in the study, tumor burden in sample, the sensitivity of the assay method, the number of scanned codons for *KRAS* mutation, or environmental factors.

The median age at diagnosis for CRC is 66 in men and 69 in women.[16] Median age at diagnosis in our group was 61 for men and 58 for women with CRC. A significant relationship was detected only between *KRAS* mutation status and age (above 70). Our results may be thought that the risk of mutation development was higher in cases above 70 years of age. No significant differences between the frequency of *KRAS* mutations and other parameters (gender, tumor location, metastatic site). Wang et al. reported that there were no statistical correlation *KRAS* mutation status and clinical parameters.[17] Another similar study of Alghamdi et al. showed correlation *KRAS* status and peritoneal metastasis.[18] The absence of a common clinical parameter associated with *KRAS* mutation status suggests that the presence of *KRAS* mutation is an independent prognostic factor.

RAS mutations were reported to be at codons 12, 13, 59, 61, 117, and 146.[19] According to the literature, the majority of the mutations occurred in codon 12 and 13 are 92-98% of all mutations), other mutations in codons 59, 61, 117, 146 (2-8% of all mutations).[20] Similar *KRAS* studies were reviewed in Table 4 and it was observed that codons 12 and 13 were scanned predominantly.

In our study, we analyze *KRAS* mutations in codons 12, 13, 59, 61, 117, and 146 by the Real-Time PCR method. We detected codon 12 mutation in 36 cases (75%), codon 13 mutation in 2 cases (4.2%), codon 61 mutation in 8 cases (16.6%) and codon 117 mutation in 2 cases (4.2%). In our study, the frequency of codon 12-13 mutations was 79.2%. Our mutation rate in codon 12-13 was observed less than other studies due to the high number of codons tested in our study.[21,22] The frequency of mutation in codon 61 (16.6%) and codon 117 (4.2%) was detected higher than similar studies.[22,23] This data emphasizes that mutations at codons except codons 12 and 13 should be tested in routine *KRAS* mutation testing.

The frequency of *KRAS* mutation was detected 45.5% in females and 49.3% in males. No significant relationship between the frequency of *KRAS* mutation and gender was found.

The distribution of KRAS mutation rates according to tumor sublocalization were 39.6% in the sigmoid colon, 20.8% in the rectum, 18.7% in ascending colon, 6.3% in the transverse colon, 4.2% in descending colon, 4.2% in the cecum. The mutation rate of *KRAS* in tumor cells in the rectum and colon were 46% and 54%, respectively. Similarly with our results, Thuc et al found the prevalence of *KRAS* mutation as 45.7% in the rectum and 54.3% in the colon.[24]

The *KRAS* G12V mutation was the most common among all mutations. The frequency of G12V mutation was 25% all of *KRAS* mutations. Followed by, G12D (23%), Q61H/Q61H/Q61L (16,6%) and G12C (14,5%) were detected. KRAS G12D and G12V mutation are the most common at codon 12, G13D, and G13C at codon 13. [25]

PCR based methodology has been widely used in *RAS* mutation testing. We also used Real-Time PCR method for codons 12, 13, 59, 62, 117, and 146. Additionaly, it is noticable that sequencing-based methods (pyrosequencing, sanger sequencing, next gene sequencing) are prefered in *KRAS* testing. Next gene sequencing allows to reveal all clinical relevant KRAS mutations with lower sensitivity than PCR-based methods. In one study, Nishi Kothari et al. examined *KRAS* mutation analysis in CRC cases with both standard *KRAS* mutation testing method and next-generation sequencing and found overall concordance as 96.1%. [26]

In Tol et al. study, the *KRAS* mutation status was assessed both by using sequencing and the Real-Time PCR-based assay. The overall concordance rate was 95.3% between the two methods.[27] Dobre et al. examined *KRAS* mutation in CRC patients by pyrosequencing and found codon 12 mutations at a rate of 79.3%.[20] Hamzehzadeh et al. used Sanger sequencing and reported that *KRAS* G12V and G12D mutations were the most common mutations.[13] Although both methods were different, the findings were consistent with our results. These rewieved data supported that Real-Time PCR method was still effective for *RAS* mutation testing.

We used FFPE tissue for genomic DNA extraction in mutation analysis. Circulating tumor DNA has also been used for analysis in some studies.[28] However, circulating tumor cell (CTC) counts were lower in CRC compared to other cancers. Because many CTCs released from the tumor area are retained in the liver before arriving the systemic circulation. In our study, the rate of liver metastasis was found 61% and consistent with this data. Mutation rates and liver metastasis rates showed that DNA extraction from FFPE tissue is useful for the accuracy of analysis method.

EGFR monoclonal antibodies have been used as chemotherapeutic agents when surgical intervention is not sufficient in the treatment of mCRC. Unfortunately, a tumor with a mutant *RAS* gene are unable to receive response to *EGFR*–targeted because of triggerred resistance.[29] New treatment combinations and agents have been tried for KRAS-mutated patients due to the high frequency and negative effects on prognosis and treatment of KRAS mutations.[30]

In 2013, Ostrem, Shokat and colleagues developed molecules that bind irreversibly and covalently to the KRAS G12C cysteine residue. With the discovery of the KRAS G12C switch II pocket, it has been possible to develop KRAS G12C driven targeted therapy.[31] Sotorasib (Lumakras) became the first FDA-approved drug to directly targeted to KRAS mutation in May 2021 and adagrasib trials followed in June 2021. However, as in other clinical studies involving many targeted therapies, adaptive resistance was observed in cancer patients who received single agent treatment with sotorasib and adagrasib.[32,33] As a result of all, the necessity of developing combination therapies has emerged to overcome these adaptive resistance mechanisms against KRAS G12C inhibitors. [34]

However, the studies showed that the response to anti-EGFR treatment was about 40-60% in *RAS* wild-type cases.[35] *BRAF, PIK3CA* and *RAS* are members of the same signaling

pathway. Also, *BRAF* or *PIK3CA* mutations were associated with resistance to anti-EGFR therapy. *BRAF* V600E mutations occured in 10-20% of CRC and were usually associated with poorer prognosis.[36] In study of Sideris and colleagues detected 17,6% *BRAF* V600E mutation in CRC cases.[37] *PIK3CA* exon 9/20 mutations occur in 15-20% of CRC and associated other molecular alterations in CRC.[38,39] We did not tested mutation status of *BRAF*, and *PIK3CA* genes. But reported data underlined that *BRAF*, *PIK3CA* should be screened for predicting the response to anti-*EGFR* therapy.[36,38,39]

Limitations

The most important limitation of our study is the scarcity of clinical data. This limited clinical data may negatively affect clinical correlations through our statistical calculations.

Conclusion

In our study, we presented the frequencies and patterns of *KRAS/NRAS* mutations of 100 CRC patients in the region. Our mutation frequencies and distributions showed that our *KRAS* mutation test method is effective for routine *KRAS* mutation testing. Effective *KRAS* mutation testing aids to select suitable patients for anti-EGFR therapy and help to get the *KRAS* to be actionable molecular target.

Conflict of Interest

All authors have no conflicts of interest to disclose.

Compliance with Ethical Statement

The study numbered KÜ GOKAEK 2017/3.7¬2017.03.01_2017/32 was approved by the Ethics Committee of Kocaeli University and followed the principles of the Declaration of Helsinki.

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Author Contributions

HS, DÇ: Conception; NÇ, DSA, SEK: Design; NÇ, SEK: Supervision; DÇ: Materials; EG, NS, GD: Data Collection; EG, NS, DSA: Analysis; GD, EG: Literature Review; EG, SEK: Writing; HS, NÇ: Critical Review

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