



A Cross-Sectional Study for Evaluation of KRAS and BRAF Mutations by Reverse Dot Blot, PCR-RFLP, and Allele-Specific PCR Methods Among Patients with Colorectal Cancer

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Abstract

Background: *KRAS* and *BRAF* genes are the biomarkers in Colorectal Cancer (CRC) which play prognostic and predictive roles in CRC treatment. Nowadays, the selection of rapid and available methods for studying *KRAS* and *BRAF* mutations in anti-EGFR therapy of patients suffering from CRC plays a significant role. In this study, the mutations of these two oncogenes were evaluated by different methods.

Methods: This study was performed on 50 Formalin-Fixed Paraffin-Embedded (FFPE) tissue blocks of patients diagnosed with colorectal cancer. After DNA extraction, *KRAS* and *BRAF* gene mutations were evaluated using reverse dot blot, and results were compared with PCR-RFLP and allele-specific PCR for *KRAS* and *BRAF* mutations, respectively.

Results: *KRAS* gene mutations were detected in 42% of patients, of which 30% were in codon 12 region, and 12% in codon 13. The most frequent mutations of *KRAS* were related to G12D and 10% of patients had *BRAF* mutated genes. The type of *KRAS* gene mutations could be evaluated by reverse dot blot method. In general, the results of PCR-RFLP and allele-specific PCR were similar to the findings by reverse dot blot method.

Conclusion: These findings suggest that PCR-RFLP and allele-specific PCR methods are suitable for screening the presence of the mutations in *KRAS* and *BRAF* oncogenes. In fact, another method with more sensitivity is needed for a more accurate assessment to determine the type of mutations. Due to higher speed of detection, reduced Turnaround Time (TAT), and possible role of some *KRAS* point mutations in overall survival, reverse dot blot analysis seems to be an optimal method.

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Introduction

Colorectal Cancer (CRC) is the third most common cancer in men, the second most common one in women, and the fourth most common one worldwide¹. Despite advancements in the diagnosis and treatment of CRC, approximately 35% of patients with CRC show stage IV or metastasis of the disease, and about 20 to 50% of patients show stage II and III of disease². The development of colorectal cancer is a multi-stage process that is associated with genetic changes³. The transformation of a normal colonic epithelial cell into a cancer cell is characterized by the activation of onco-

genes and the inactivation of tumor suppressor genes⁴. Different molecular mechanisms are involved in the biological course of colorectal cancer development. In the genetic pathway, the conversion of normal cells to the proliferative epithelial cells is associated to the mutations in Adenomatous Polyposis Coli (*APC*) gene and the conversion of proliferative epithelial cells to an adenoma is also linked to changes in DNA methylation. At the adenoma stage, mutations in the *BRAF* and *KRAS* genes occur. Finally, adenoma transformation into carcinoma is associated with a mutation in the *P53*

gene⁵. KRAS protein is a small GTPase which plays an important role in the transduction of intracellular signals. This gene is a member of MAP kinase pathway⁶. The mutation in KRAS is capable of reducing this gene's protein activity and thus decreases GTPase activity⁷. The prognostic and predictive role of KRAS was determined in literature by studying on this oncogene. It also encodes the cytoplasmic serine-threonine kinase, BRAF gene, which immediately activates MAPK signaling pathway downstream of KRAS. Abnormal activation of BRAF gene causes cell proliferation and survival which are important for tumorigenesis in many types of tumors⁸. The role of KRAS and BRAF mutations in survival of CRC patients and response to standard chemotherapy are still controversial issues. Therefore, the study of these mutations is of great importance for patients with CRC. A study by Garcia-Carbonero *et al* on 792 CRC patients identified KRAS and BRAF genes as prognostic biomarkers as well as biomarkers of standard first-line chemotherapy response in metastatic colon cancer⁹. In CRC management, detecting the absence of KRAS, BRAF, NRAS, and PIK3CA gene mutations with the choice of anti-EGFR therapy is one of the most useful strategies¹⁰. Due to the high importance and role of these two oncogenes as CRC biomarkers and advancements in technology, different methods were developed to study and evaluate KRAS and BRAF mutations. Next-generation sequencing, real-time PCR, PCR-RFLP, allele-specific PCR (ASPCR), pyrosequencing, reverse dot blot, sequenom genotyping assays, LNA-PCR sequencing, HRM sequencing, multiplex mutation assay, and HRM-SNaPshot are a short list of different techniques for detecting KRAS and BRAF mutations. In 2016, Matsunaga *et al* compared the result of KRAS mutations by direct sequencing, Scorpion-ARMS assays, pyrosequencing, and Luminex xMAP methods¹¹. Nagakubo *et al* compared different methods (Sanger sequencing, PCR-reverse sequence-specific oligonucleotide probe, and next-generation sequencing) to detect KRAS, BRAF, and NRAS mutations¹².

Among these different methods, reverse dot blot, PCR-RFLP, and allele-specific PCR were used to perform this experiment. First, reverse dot blot was performed to detect KRAS and BRAF mutations and results were compared by PCR-RFLP and allele-specific PCR for KRAS and BRAF mutations, respectively. The mechanism of PCR-restriction fragment length polymorphism (PCR-RFLP) is based on discovering a specific recognition site in a target sequence¹³. PCR-RFLP is a method which uses restriction enzymes to digest DNA, and the digested fragments will be loaded on the gel. In this method, if there are genetic changes or a mutation, it appears as a change in the size of one or two fragments in the gel electrophoresis due to insertion or deletion¹⁴. Allele-specific PCR method has been used to detect Single Nucleotide Polymorphisms (SNPs) or bands of wild-type and mutant alleles, and

the PCR product will be loaded on the gel. Reverse allele-specific oligonucleotide assay (Reverse dot blot) is a method for the molecular characterization of disorders with high mutation spectrum¹⁵. In reverse dot blot hybridization, after a multiplex PCR, PCR products are hybridized to wild-type and mutant oligonucleotide probes on a nitrocellulose membrane¹⁴, and then the positive and control bands will appear on a nitrocellulose paper.

Materials and Methods

In this cross-sectional study, 50 FFPE tissue blocks belonging to patients with colorectal cancer with a mean age of 56.9 years (34 males and 16 females) were randomly collected from cases who referred to Payvand clinical laboratory from different parts of the country. Each patient completed a questionnaire about age, gender, city of residence, and treatment history, and before extraction, the blocks were checked by each patient's pathology report.

Genomic DNA extraction

To investigate the mutations of KRAS and BRAF genes, genomic DNA was extracted from FFPE tissue blocks using High Pure PCR Template Preparation Kit (Roche Life Science, Germany). The purification of desired DNA was calculated by determining the optical absorption ratio of 260 OD/280 OD and its concentration using the formula, $\mu\text{g/ml}=50\times\text{P}\times\text{OD}_{260}$ (DNA dilution grade=P), by Biophotometer; moreover, to ensure the health of DNA, polymerase chain reaction with a housekeeping gene such as β -globin was performed. DNAs that did not pass the quality control stage and did not have a light absorption ratio of 1.6 to 2 and also a minimum concentration of 50 ml/ μg were excluded from the evaluation.

Investigation of KRAS and BRAF gene mutations by reverse dot blot

To perform reverse dot blot (using RDB 2250 KRAS/BRAF Kit, AID Autoimmun Diagnostika GmbH, Germany), first, the multiplex polymerase chain reaction was performed on genomic DNA. The reaction mixture consisted of 15 μl PN-mix, 2.5 μl 10 \times PCR reaction buffer, 1.5 μl MgCl₂, one unit of the single polymerase enzyme, and 5 μl DNA sample with a total volume of 25 μl .

The temperature program included an initial denaturation at 95°C for 5 min (One cycle). The polymerase reaction in the first step was performed at 95°C for 20 s, 60°C for 20 s and for 10 cycles. In the second step, it was performed at 95°C for 20 s, 55°C for 20 s, and 72°C for 20 s (26 cycles). Then, the final extension reaction was performed at 72°C for 8 min (One cycle).

For hybridization, first 20 μl of denaturation solution was mixed with 20 μl of PCR mixture, followed by incubation at room temperature for 5 min. The nitrocellulose strip was incubated in 1 ml of hybridization solution for 30 min at 47°C. The hybridization

buffer was completely removed from the strip and the strip was washed twice with stringency wash solution, each time for 1 min. The nitrocellulose strip was then incubated in 1 ml of stringency wash solution for 15 min at 47°C. The strip was washed twice at room temperature for 1 min by adding 1 ml of diluted rinse solution. Then, 1 ml of conjugate solution (Concentration of this solution was diluted 1:100 by conjugate buffer) was added to the strip, followed by incubation in a horizontal shaker at room temperature for 30 min. After removing the conjugate solution from the well, the strip was washed three times with 1 ml of rinse solution for 1 min. Afterward, 1 ml of the substrate solution, which reached room temperature, was added to the strip, followed by incubation for 10 to 20 min. After this period, the reaction was stopped by adding 1 ml of distilled water. After drying the strip, the results were interpreted using a nitrocellulose membrane scan.

Evaluation of KRAS gene mutations by PCR-RFLP method

Specific primers were designed and synthesized to identify codons 12 and 13 of the KRAS proto-oncogene using the NCBI bank and Blast software (RAS A: 5'-ACTGAATATAAACTTGTGGTCCATGGAGCT-3', RAS B: 5'-TTATCTGTATCAAAGAATGGTCCTGCACCA-3', and RAS C: 5'-GGATGGTCCTCCACCA GTAATATGGATATT A-3').

RAS A and RAS B primers were designed to synthesize 166 bp fragments in the first PCR and also RAS C primer along with RAS A primer was used to synthesize the second PCR product.

The first and second reaction mixtures consisted of 12.5 µl of amplicon master mix, 1 µl of each RASA (15 pmol) and RASB (15 pmol) primer for the first reaction mixture, 1 µl of each RASA (15 pmol) and RASC (15 pmol) primer for a mixture of the second reaction, and genomic DNA up to a concentration of 100 ng. The final volume of 25 µl was prepared.

The PCR reaction consisted of initial denaturation at 95°C for 4 min (One cycle), 30 cycles at 94°C for one min, 50°C for 1 min, and 72°C for 2 min. The final extension was performed in a cycle at 72°C for 5 min. To detect mutations in codons 12 and 13, the first PCR product was enzymatically digested with BSTX-I and XcmI enzymes (New England Biolabs, USA) for 24 hr according to the standard protocol. Also, to identify mutations in codons 12 and 13 of the second PCR product, enzymatic digestion was performed using BSTX-I and XcmI enzymes for 24 hr, respectively. The results of enzymatic digestion were evaluated on 12% polyacrylamide gel and by silver nitrate staining.

Evaluation of BRAF gene mutations by allele-specific PCR

In this method, two series of primers were used to detect the V600E mutation BRAF (F-wild type): 5'-TAGGTGATTTTGGTCTAGCTACCGT- 3', BRAF (Reverse): 5'-GTA ACTCAGCAGCATCTCAGGG-3', BRAF (F-mutant type): 5'-TAGGTGATTTTGGTCTAGCTACCGA- 3'). Accordingly, two separate mixtures were prepared for PCR.

The first and second reaction mixtures consisted of 12.5 µl of the master mix, one µl of each BRAF (F-wild type, 15 pmol) and BRAF (Reverse, 15 pmol) primer for the first reaction mixture and one µl of each ready-to-use primer BRAF (F-mutant type, 15 pmol) and BRAF reverse, 15 pmol) for a mixture of the second reaction. Furthermore, genomic DNA was prepared up to a concentration of 100 ng and a final volume of 25 µl was prepared.

The PCR reaction consisted of initial denaturation at 95°C for 5 min (One cycle), 38 cycles at 95°C for 40 s, 58°C for 1 min, and 72°C for 20 s. The final extension was performed for one cycle at 72°C for 10 min. The reaction product was examined on 12% polyacrylamide gel and by silver nitrate staining.

Statistical analysis

The results were analyzed by SPSS software version 19 (IBM, USA). The chi-square test was applied to investigate the relationship between mutations and clinicopathological factors. The correlation test was used to investigate the relationship between mutations and factors with the normal distribution and p<0.05 was considered statistically significant.

Results

Results of reverse dot blot

In the assessment of KRAS and BRAF gene mutations by reverse dot blot, each nitrocellulose strip had internal PCR control and conjugated buffer control for controlling hybridization; if there was no mutation in both oncogenes, only the band control could appear. If there was a mutation, the same area of the band could be observed depending on the type of point mutation (Figure 1). Due to transition and transgenic mutations, the wild-type sequence of GGT in codon 12 was mutated into c.35G> C (p.G12A), c.34G> C (p.G12R), c.35G> A (p.G12D), c.34G> T (p.G12C), c. [34G> A; 35G> T] (p.G12I), c. [34G> C; 35G> T] (p.G12I), c.34G> A (p.G12S), and c.35G> T (p.G12V).

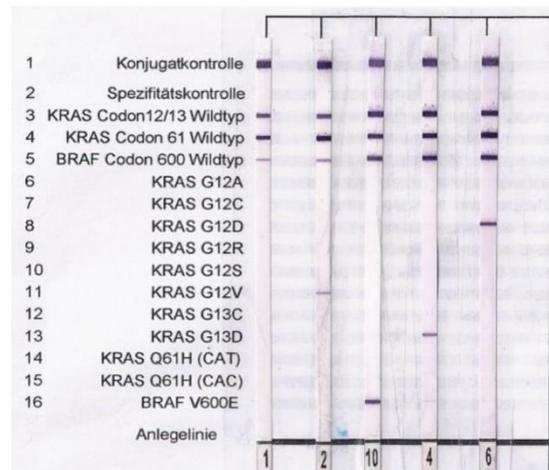


Figure 1. Nitrocellulose strips from reverse dot blotting. Results from right: G12D mutation, G13D mutation, V600E mutation, G12V mutation, no mutation in both oncogenes.

Evaluation of KRAS and BRAF Genes by Various Methods

Table 1. Correlation of mutation status with clinical and histopathological features

Mutation	Frequency of mutation (number)	Pearson Chi-Square test									
		Sex	Cecum (n=5; 10%)	Colon (n=33; 66%)	Rectum (n=7; 14%)	Liver (n=5; 10%)	Metastasis	Mucinous	Non-mucinous	High differentiation	Low differentiation
G12A	2% (1)	0.32	-	0.66	-	-	-	-	-	-	-
G12D	16% (8)	0.495	0.176	0.558	-	0.599	-	-	-	-	-
G12C	2% (1)	0.68	-	-	-	-	-	-	-	-	-
G12S	4% (2)	0.458	-	0.431	-	-	-	-	-	-	-
G12V	6% (3)	0.305	-	0.736	0.37	-	-	-	-	-	-
G13D	12% (6)	0.365	0.487	0.674	-	0.487	-	-	-	-	-
V600E	10% (5)	0.209	-	0.44	0.546	-	-	-	-	-	-
KRAS Codon 12	-	-	-	-	-	-	0.5	0.589	0.507	0.354	0.574
KRAS Codon 13	-	-	-	-	-	-	0.5	0.545	0.245	0.415	0.627
BRAF Mutation	-	-	-	-	-	-	-	0.5	0.243	0.524	0.229

The highest frequency of mutations was related to G12D (16%), and G12I and G12R mutations were not found in the study population. The frequency of mutations found in this study is shown in table 1.

Regarding mutations in codon 13 of the KRAS gene (Wild type GGC sequence), mutations of c.38G> A (p.G13D) and c.37G> T (p.G13C) were observed and G13C mutation was not found in this study; data shows the frequency of G13D mutation is equal to 12% (Table 1).

Results of PCR-RFLP

PCR-RFLP results about KRAS gene revealed the presence of mutations in the codons 12 and 13 of some samples of the studied patients. None of the enzymes could break the fragment if the fragment from the first PCR had a mutation. Fragments of 28 bp and 138 bp appeared by enzymatic digestion reaction in wild-type sequence. Moreover, if the amplified fragment from the second PCR had a mutation, three fragments of 134 bp, 106 bp, and 18 bp could be produced from the second PCR in mutable sequences, while bp18, bp106, and 28 bp fragments could be produced as a result of enzymatic digestion in normal type sequences (Figure 2). As shown in figures 2 and 3, 30% of samples showed a mutation in codon 12, and 12% of them had a mutation in codon 13 of KRAS oncogene.

Results of allele-specific PCR

To detect BRAF gene mutations, the presence of the allele of normal type and mutant type was investigated. Figure 4 shows an example of polyacrylamide gel obtained from this study (Figure 4), in which the wild type and mutant alleles had a band at 180 bp position, and the interpretation of findings were done based on the results obtained from both types of alleles. Examining the frequency of V600E mutation in the BRAF

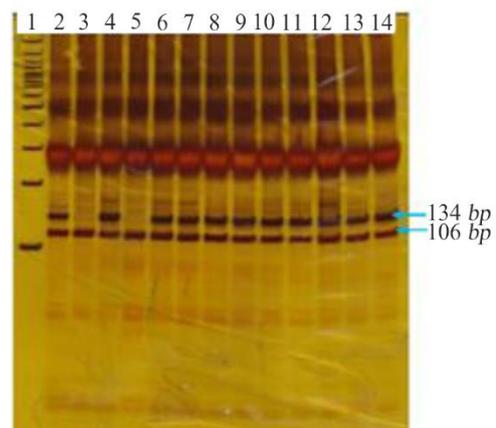


Figure 2. Polyacrylamide gel. Codon 12 KRAS has two bands at the 134 bp and 106 bp sites, indicating a mutation. Bands at 106 bp had no mutations in this codon: Lane 1: a 100 bp marker, Lane 3 and Lane 5: no mutations, and other lanes: mutations.

gene revealed that out of 50 samples studied, 90% did not have V600E mutation, and only 10% showed V600E mutation.

Concordance of mutations analysis

The frequency of mutations found in codons 12 and 13 by the reverse dot blot method was consistent with the PCR-RFLP results, and also the results of V600E mutation in BRAF by the allele-specific PCR and reverse dot blot method were similar. A comparison of results among different methods is described in table 2. In this table, the differences between the methods in the evaluation of mutations are indicated. In reverse dot blot method, 10 point mutations in KRAS gene were examined by a mutation in the BRAF gene, and in addition to codons 12 and 13, codon 61 mutations in the KRAS gene were also examined. However, in RFLP

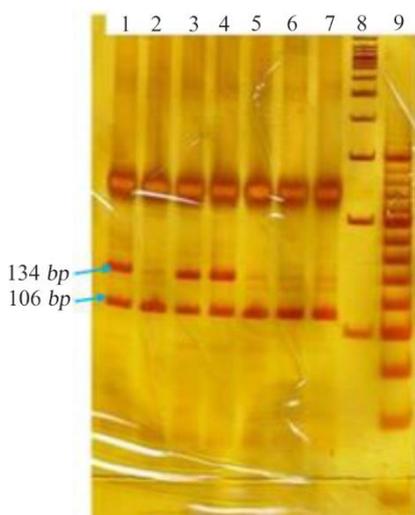


Figure 3. Polyacrylamide gel of the 13 oncogene KRAS codon. Dual-band samples at bp134 and bp106 sites have mutations in this codon, and bands at 106 bp site have no mutations in this codon: Lane 9: 20 bp marker, Lane 8 100 bp marker, Lanes 5, 6, and 7: no mutations and Lanes 1, 3, and 4: mutations.

method, only mutations in codons 12 and 13 were evaluated and mutation evaluation in *BRAF* gene was confirmed by allele-specific method.

Results of statistical analysis

Out of 50 samples, five had metastatic liver tissue of CRC origin, and other samples of cecum, colon, and rectum tissues had CRC (Table 1); out of 50 samples, 29 (58%) tumors metastasized, and in 21 (48%) samples, metastasis did not occur.

The relationship between sex and each of the mutations in *KRAS* and *BRAF* genes was determined using the Pearson's chi square test. There was no significant relationship between sex and type of mutations in these two genes.

Regarding this analysis, no significant relationship was found among the mutations in *KRAS* and *BRAF*

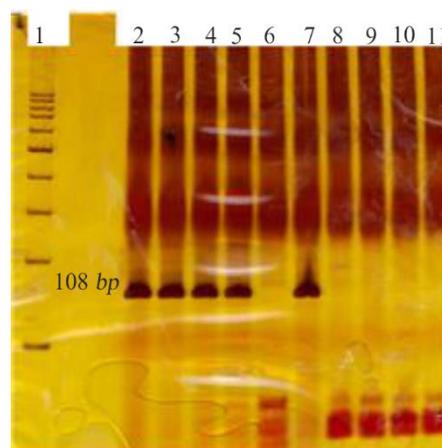


Figure 4. *BRAF* gene polyacrylamide gel. Lane1: 100 bp marker, Lane 2 to Lane 6: mutant alleles, and Lane 7 to Lane 11: non-mutant alleles. It is interpreted as meaning that the first sample has a mutation and the other three samples do not have a mutation. Lane 6 and 11 are PCR (M.M) mixture contamination controls.

genes and metastasis at the time of diagnosis ($p>0.05$). No significant relationship was found among the mutations in codon 12 of *KRAS* gene with high and low differentiation ($p>0.05$), and chi-square test showed no significant relationship among the mutations in *KRAS* 12 gene with mucinous and non-mucinous tumors. The results showed no significant relationship among the mutations in 13 *KRAS* gene with mucinous and non-mucinous tumors.

There was no significant association among the mutations in *BRAF* gene with mucinous and non-mucinous tumors.

Discussion

KRAS oncogene is an important member of EGFR signaling cascade family, and *KRAS* activating mutations are among the most common mutations in human cancers¹⁶. Due to the high prevalence of colorectal

Table 2. Comparison of results among different methods

Mutations	Methods		
	Reverse dot blot (n/%)	PCR-RFLP (n/%)	Allele specific PCR (n/%)
G12A	1/2%	-	-
G12D	8/16%	-	-
G12C	1/2%	-	-
G12R	-	-	-
G12S	2/4%	-	-
G12V	3/6%	-	-
G13C	-	-	-
G13D	6/12%	-	-
KRAS Q61H (CAT)	-	-	-
KRAS Q61H (CAC)	-	-	-
Point mutation in codon 12 KRAS	15/30%	15/30%	-
Point Mutation in codon 13 KRAS	6/12%	6/12%	-
BRAF V600E	5/10%	-	5/10%

cancer in the world and the role of KRAS codons 12 and 13 in malignancy and tumor progression, timely screening is essential to identify patients at the early stages. Determining the type of mutation using non-invasive and rapid methods is also of considerable importance for targeted therapies appropriate to the type of mutation¹⁷. The mutations in the codons 12 and 13 lead to changes in RAS protein which is resistant to GTPase activity; also, they result in long-term persistence of active form bound to GTP, thus leading to intensified cell growth and proliferation. BRAF and KRAS mutations both occur at the early stages of colorectal cancer but are rarely seen together¹⁸. In a study by Velho *et al* in Portugal, Li *et al* in China, and Brink *et al* on Dutch patients, 35.3%, 37%, and 33.3% of patients had mutations in the KRAS oncogene, respectively¹⁹⁻²¹. The most common mutations in KRAS gene are in codon 12 and include the amino acid substitutions, G13D, G12S, and G12D, of which G12D mutations are the most common²². These results are consistent with results of our study because the highest frequency of amino acid substitution was found to be related to G12D (16%). In a study by Garcia-Carbonero *et al* on KRAS and BRAF mutations as predictors and predictive biomarkers in metastatic colon cancers, COX regression analysis showed that KRAS and BRAF in metastatic tumors are important agents for survival. Garcia-Carbonero *et al* stated that patients with KRAS mutations had a poorer response to chemotherapy²³.

In thyroid cancer, BRAF mutations were found to upregulate microenvironment genes, potentially increasing tumor invasion. The frequency of BRAF mutations is high in melanoma (44%) and lower in thyroid, colorectal, and ovarian cancers (8, 6, 5%, respectively)²⁴. In our study, the frequency of KRAS and BRAF oncogenes mutations in a population of CRC patients with a mean age of 56.96 was found to be 42% (30% in codon 12 and 12% in codon 13) and 10%, respectively. The highest number of samples with FFPE blocks belonged to male patients (68%, with a mean age of 56 years), while the lowest number of samples belonged to female patients (34%, with a mean age of 54.8 years) which is consistent to the study of Li *et al*. Among these patients, 15 had mutations in codon 12 of KRAS gene (30%), while 6 patients had mutations in codon 13 (12%).

Due to the results of this research and other studies, the highest frequency of mutations is related to codons 12 and then 13, although several studies indicated the role of codons 61 and 146 and their mutations such as codons 12 and 13; however, the frequency of mutations in codon 61 and 146 is lower than codons 12 and 13. Therefore, codons 61 and 146 also have a prognostic role in the treatment of patients with CRC, but the frequency of these mutations is very low compared to codons 12 and 13 which is consistent with our study findings showing the high frequency of mutations in

codon 12 and also the low frequency of mutations in codon 61²⁵. The frequency of KRAS gene mutations in CRC patients is approximately 30 to 46% based on previous articles.

This wide range of frequencies reported from KRAS mutations is possible due to various factors, including the sensitivity and specificity of mutation detection methods, the small number of patients, or the variability in the region in which the gene was selected, namely codons 12, 13 and/or 61 regions²⁶.

Regarding the role of these two genes in improving CRC patients' outcomes, extensive studies have been performed to quickly and accurately evaluate these two genes in recent decades.

Techniques used for the mutant screening such as temperature gradient gel electrophoresis or SSCP and methods for mutant detection such as PCR-RFLP or allele-specific PCR amplification (MASA) may show differences in the sensitivity and/or mutation detection characteristics of the KRAS gene²⁷. Though the mutations were studied in a small population of patients, the selection of these patients was random and results were investigated by comparing three methods of reverse dot blot, PCR-RFLP, and allele-specific PCR.

The hybridization method was used to detect the mutations of codons 12, 13, 61 in the KRAS oncogene and also V600E mutation in the BRAF oncogene. Specific primers and probes were designed for each point mutation to detect them. Moreover, each nitrocellulose strip had internal controls for PCR accuracy and hybridization steps. However, all samples were re-analyzed by RFLP for the KRAS gene and allele-specific PCR for the BRAF gene. The results of these two methods confirmed each other, and the samples with mutations in each method were well distinguishable from the samples without mutations.

In this study, the mutations including G12A, G12R, G12D, G12C, G12S, and G12V in codon 12 and G13D, G13C in codon 13, Q61H in codon 61 of KRAS proto-oncogene, and V600E mutation in BRAF proto-oncogene were analyzed by reverse dot blot method. The mutations such as c.183A> C (p.Q61H), c.183A> T (p.Q61H), c.37G> T (p.G13C), and c.34G> C (p.G12R) were not found, and the highest frequency was related to G12D mutation (16%) of codon 12, which was studied and evaluated by Ince *et al* in 2005²⁸, Cabart *et al* in 2016²⁹, and Ergun *et al* in 2019³⁰. In the reverse dot blot method, point mutations in KRAS oncogene and also V600E mutation in BRAF oncogene were evaluated, and in the PCR-RFLP method, only the presence of mutations in codons 12 and 13 of KRAS was examined. The allele-specific PCR method was used to detect BRAF mutation in CRC. Hatzaki *et al* in 2001 presented a modified mutagenic PCR-RFLP method for the KRAS codon exon 1 mutations in tumor samples from NSCLC patients³¹.

Some studies have reported the importance of detecting KRAS point mutations in CRC patients. De

Roock *et al* and Tejpar *et al* reported that some specific mutations in *KRAS* can affect patients' outcomes and patients with a mutation in codon 13 (G13D) have a survival benefit^{32,33}. Studies of Al-Mulla *et al*, Guerrero *et al*, and Smith *et al* on *KRAS* codon 12 mutations shows that patients with mutations in codon 12 compared to codon 13 may demonstrate carcinogenic potential as a result of apoptosis inhibition, loss of contact inhibition, and also increased contact-independent growth³⁴⁻³⁶.

KRAS mutations have a prognostic role in CRC patients; in general, an association between *KRAS* codon 12 mutation (Specific G12V, G12C mutations) and overall survival was illustrated in patients with recurrent and metastatic colorectal cancer and wild type *KRAS* shows an increased overall survival³⁷.

KRAS G12D mutation shows a poorer prognostic effect on patients compared to the other mutations³⁸, and *KRAS* G12V shows a biological behavior in CRC patients³⁹.

Kaelin reported the synthetic lethality of *KRAS*, in which the mutations in two or more genes cause cell death⁴⁰ which is considered an advantage that leads to increased cytotoxicity against cancer cells without the toxicity associated with current chemotherapy⁴¹.

Although the frequency of *KRAS* mutations in the hybridization report did not differ from PCR-RFLP results, point mutations in each codon were excluded in this study. Detecting point mutations is important in overall survival of patients. Besides, another method is required to examine *BRAF* mutation as the process of mutation analysis in *KRAS* and *BRAF* is very time-consuming.

As a result, the reverse dot blot method is a more sensitive and specific method to evaluate mutations in these oncogenes and PCR-RFLP and allele-specific PCR methods seem to be suitable for screening the existence of mutation in *KRAS* and *BRAF* oncogenes.

Allele-specific PCR is an optimal method to evaluate V600E mutation in other cancers in which *BRAF* alone is a biomarker for detecting melanoma, thyroid, and ovarian cancer. In the studies of Osumi *et al*⁴² and Chen *et al*⁴³, the effect of mutation type on anti-EGFR therapies was investigated; therefore, point mutation analysis plays an essential role in treating patients with CRC.

Conclusion

Due to high frequency of mutations, the young age at diagnosis, and the oncogenes' roles in disease management, it is recommended to study the mutations of *KRAS* and *BRAF* oncogenes in patients with colorectal cancer. The results of PCR-RFLP and allele-specific PCR were similar to reverse dot blot, although PCR-RFLP and allele-specific PCR are time-consuming methods. These findings suggest that PCR-RFLP and allele-specific PCR as confirmatory methods are suitable for screening the existence and presence of the

mutation in *KRAS* and *BRAF* oncogenes. Furthermore, for a more accurate assessment and also discovering the possible role of the type of mutations, another method with more sensitivity is needed. In general, the reverse dot blot method was faster and more sensitive to detect *KRAS* and *BRAF* mutations; moreover, type of *KRAS* mutations could be assessed which saves response time, reduces additional costs, and provides higher quality services. Therefore, in the current study, the superiority of the reverse method over the RFLP method was proved.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin* 2019;69(1):7-34.
2. Van Abbema D, Vissers P, de Vos-Geelen J, Lemmens V, Janssen-Heijnen M, Tjan-Heijnen V. Trends in overall survival and treatment patterns in two large population-based cohorts of patients with breast and colorectal cancer. *Cancers (Basel)* 2019;11(9):1239.
3. Lubomierski N, Plotz G, Wormek M, Engels K, Kriener S, Trojan J, et al. *BRAF* mutations in colorectal carcinoma suggest two entities of microsatellite-unstable tumors. *Cancer* 2005;104(5):952-61.
4. Zacharakis M, Xynos ID, Lazaris A, Smaro T, Kosmas C, Dokou A, et al. Predictors of survival in stage IV metastatic colorectal cancer. *Anticancer Res* 2010;30(2): 653-60.
5. Benavides M, Díaz-Rubio E, Carrato A, Abad A, Guillén C, Garcia-Alfonso P, et al. Tumour location and efficacy of first-line EGFR inhibitors in *KRAS*/RAS wild-type metastatic colorectal cancer: retrospective analyses of two phase II randomised Spanish TTD trials. *ESMO Open* 2019;4(6):e000599.
6. Solassol J, Vendrell J, Märkl B, Haas C, Bellosillo B, Montagut C, et al. Multi-center evaluation of the fully automated PCR-based Idylla™ *KRAS* mutation assay for rapid *KRAS* mutation status determination on formalin-fixed paraffin-embedded tissue of human colorectal cancer. *PLoS One* 2016;11(9):e0163444.
7. Ogino S, Meyerhardt JA, Irahara N, Niedzwiecki D, Hollis D, Saltz LB, et al. *KRAS* mutation in stage III colon cancer and clinical outcome following intergroup trial CALGB 89803. *Clin Cancer Res* 2009;15(23): 7322-9.
8. Midhun L, Shaheen S, Deisch J, Senthil M, Tsai J, Hsueh CT. Concomitant *KRAS* and *BRAF* mutations in colorectal cancer. *J Gastrointest Oncol* 2019;10(3):577-81.
9. Garcia-Carbonero N, Martinez-Useros J, Li W, Orta A, Perez N, Carames C, et al. *KRAS* and *BRAF* mutations

as prognostic and predictive biomarkers for standard chemotherapy response in metastatic colorectal cancer: a single institutional study. *Cells* 2020;9(1):219.

10. Vacante M, Borzi AM, Basile F, Biondi A. Biomarkers in colorectal cancer: Current clinical utility and future perspectives. *World J Clin Cases* 2018;6(15):869-81.
11. Matsunaga M, Kaneta T, Miwa K, Ichikawa W, Fujita KI, Nagashima F, et al. A comparison of four methods for detecting KRAS mutations in formalin-fixed specimens from metastatic colorectal cancer patients. *Oncol Lett* 2016;12(1):150-6.
12. Nagakubo Y, Hirotsu Y, Amemiya K, Oyama T, Mochizuki H, Omata M. Accurate detection of KRAS, NRAS and BRAF mutations in metastatic colorectal cancers by bridged nucleic acid-clamp real-time PCR. *BMC Med Genomics* 2019;12(1):162.
13. Hashim HO, Al-Shuhaib MB. Exploring the potential and limitations of PCR-RFLP and PCR-SSCP for SNP detection: a review. *J Applied Biotechnology Reports* 2019;6(4):137-44.
14. Bluth MJ, Bluth MH. Molecular pathology techniques: Advances in 2018. *Clin Lab Med* 2018;38(2):215-36.
15. Gold B. Origin and utility of the reverse dot-blot. *Expert Rev Mol Diagn* 2003;3(2):143-52.
16. Hayama T, Hashiguchi Y, Okamoto K, Okada Y, Ono K, Shimada R, et al. G12V and G12C mutations in the gene KRAS are associated with a poorer prognosis in primary colorectal cancer. *Int J Colorectal Dis* 2019;34(8):1491-6.
17. Yang MM, Wang J, Dong L, Teng Y, Liu P, Fan JJ, et al. Lack of association of C3 gene with uveitis: additional insights into the genetic profile of uveitis regarding complement pathway genes. *Sci Rep* 2017;7(1):879.
18. Li W, Liu Y, Cai S, Yang C, Lin Z, Zhou L, et al. Not all mutations of KRAS predict poor prognosis in patients with colorectal cancer. *Int J Clin Exp Pathol* 2019;12(3): 957-67.
19. Velho S, Moutinho C, Cirnes L, Albuquerque C, Hamelein R, Schmitt F, et al. BRAF, KRAS and PIK3CA mutations in colorectal serrated polyps and cancer: primary or secondary genetic events in colorectal carcinogenesis?. *BMC Cancer* 2008;8(1):255.
20. Li Z, Chen Y, Wang D, Wang G, He L, Suo J. Detection of KRAS mutations and their associations with clinicopathological features and survival in Chinese colorectal cancer patients. *J Int Med Res* 2012;40(4): 1589-98.
21. Brink M, de Goeij AF, Weijenberg MP, Roemen GM, Lentjes MH, Pachen MM, et al. K-ras oncogene mutations in sporadic colorectal cancer in The Netherlands Cohort Study. *Carcinogenesis* 2003;24(4):703-10.
22. Van Cutsem E, Kohne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, et al. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011;29(15):2011-9.
23. Garcia-Carbonero N, Martinez-Useros J, Li W, Orta A, Perez N, Carames C, et al. KRAS and BRAF mutations

as prognostic and predictive biomarkers for standard chemotherapy response in metastatic colorectal cancer: A single institutional study. *Cells* 2020;9(1):219.

24. Janku F, Lee JJ, Tsimberidou AM, Hong DS, Naing A, Falchook GS, et al. PIK3CA mutations frequently coexist with RAS and BRAF mutations in patients with advanced cancers. *PLoS One* 2011;6(7):e22769.
25. Chiu JW, Krzyzanowska MK, Serra S, Knox JJ, Dhani NC, Mackay H, et al. Molecular profiling of patients with advanced colorectal cancer: princess margaret cancer centre experience. *Clin Colorectal Cancer* 2018;17(1):73-9.
26. Loupakis F, Ruzzo AN, Cremolini C, Vincenzi B, Salvatore L, Santini D, et al. KRAS codon 61, 146 and BRAF mutations predict resistance to cetuximab plus irinotecan in KRAS codon 12 and 13 wild-type metastatic colorectal cancer. *Br J Cancer* 2009;101(4):715-21.
27. Hutchins G, Southward K, Handley K, Magill L, Beaumont C, Stahlschmidt J, et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. *J Clin Oncol* 2011;29(10):1261-70.
28. Ince WL, Jubb AM, Holden SN, Holmgren EB, Tobin P, Sridhar M, et al. Association of k-ras, b-raf, and p53 status with the treatment effect of bevacizumab. *J Natl Cancer Inst* 2005;97(13):981-9.
29. Cabart M, Frenel JS, Campion L, Ramee JF, Dupuis O, Senellart H, et al. [KRAS mutation does not influence oxaliplatin or irinotecan efficacy, in association with bevacizumab, in first line treatment of metastatic colorectal cancer]. *Bull Cancer* 2016;103(6):541-51. French.
30. Ergun Y, Acikgoz Y, Bal O, Ucar G, Dirikoc M, Caliskan Yildirim E, et al. KRAS codon 12 and 13 mutations may guide the selection of irinotecan or oxaliplatin in first-line treatment of metastatic colorectal cancer. *Expert Rev Mol Diagn* 2019;19(12):1131-40.
31. Hatzaki A, Razi E, Anagnostopoulou K, Iliadis K, Kodaxis A, Papaioannou D, et al. A modified mutagenic PCR-RFLP method for K-ras codon 12 and 13 mutations detection in NSCLC patients. *Mol Cell Probes* 2001;15(5):243-7.
32. De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, et al. Association of KRAS p. G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 2010;304(16):1812-20.
33. Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, Van Cutsem E. Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. *J Clin Oncol* 2012;30(29):3570-7.
34. Al-Mulla F, Milner-White EJ, Going JJ, Birnie GD. Structural differences between valine-12 and aspartate-12 Ras proteins may modify carcinoma aggression. *J Pathol* 1999;187(4):433-8.
35. Guerrero S, Casanova I, Farré L, Mazo A, Capellà G, Manges R. K-ras codon 12 mutation induces higher level of resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation

- or proto-oncogene overexpression. *Cancer Res* 2000;60(23):6750-6.
36. Smith G, Bounds R, Wolf H, Steele RJ, Carey FA, Wolf CR. Activating K-Ras mutations outwith 'hotspot' codons in sporadic colorectal tumours-implications for personalised cancer medicine. *Br J Cancer* 2010;102(4):693-703.
37. Jones RP, Sutton PA, Evans JP, Clifford R, McAvoy A, Lewis J, et al. Specific mutations in KRAS codon 12 are associated with worse overall survival in patients with advanced and recurrent colorectal cancer. *Br J Cancer* 2017;116(7):923-9.
38. Zlobec I, Kovac M, Erzberger P, Molinari F, Bihl MP, Ruffe A, et al. Combined analysis of specific KRAS mutation, BRAF and microsatellite instability identifies prognostic subgroups of sporadic and hereditary colorectal cancer. *Int J Cancer* 2010;127(11):2569-75.
39. Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 2001;85(5):692-6.
40. Kaelin Jr WG. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* 2005;5(9):689-98.
41. Arrington AK, Heinrich EL, Lee W, Duldulao M, Patel S, Sanchez J, et al. Prognostic and predictive roles of KRAS mutation in colorectal cancer. *Int J Mol Sci* 2012;13(10):12153-68.
42. Osumi H, Shinozaki E, Osako M, Kawazoe Y, Oba M, Misaka T, et al. Cetuximab treatment for metastatic colorectal cancer with KRAS p. G13D mutations improves progression-free survival. *Mol Clin Oncol* 2015;3(5):1053-7.
43. Chen CC, Er TK, Liu YY, Hwang JK, Barrio MJ, Rodrigo M, et al. Computational analysis of KRAS mutations: implications for different effects on the KRAS p. G12D and p. G13D mutations. *PLoS One* 2013;8(2):e55793.