

ORIGINAL ARTICLES

ARMS-PCR based diagnosis of *APCB* and *KRAS* genes in colorectal cancer patients in Iraqi Sulaimania province

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ABSTRACT

Amplification Refractory Mutation System (ARMS) assay is achieved for 30 blood samples of colorectal cancer (CRC) patients including 17 male and 13 female in order to determine the mutations in *APCB* gene and a proto-oncogene *KRAS*. ARMS-PCR analysis shows that all 30 samples are negative for I1307K variant (*APCB*) and seven common mutations in codons 12 and 13 (*APCB*). In addition two CRC patient samples are randomly chosen and purified for sequencing at Genome Center/KOYA University, then the sequence of the samples were BLAST on NCBI Website. The sequence alignment showed that both samples were wild for I1307K (*APCB*) and (G12S, G12C, G12A, G12D, G12V, G13D) (*KRAS*). We believe that these variants might absent in CRC patients of Iraqi Sulaimania province.

Key Words: Colorectal cancer, *KRAS* gene, *APCB* gene, ARMS-PCR

1. INTRODUCTION

Cancer should be considered as not one but many diseases, in which almost every single patient is genetically and clinically unique.^[1] Hence, recent research has turned to find disease markers and a deeper understanding of genetic variation of cancer related genes and to find potential targets for future therapies.

Cancer is a group of diseases that share the central property of uncontrolled cellular proliferation. It is characterized by the progressive growth of neoplastic cells, which have clonally expanded to create an abnormal tissue;^[2] Also it contains aberrations that impair normal processes within cells, causing them to gain new and pathological abilities to develop malignancy.^[3]

Colorectal cancer (CRC) ranks among the three most com-

mon cancers in terms of both cancer incidence and cancer related deaths in most worldwide.^[4] The majority of sporadic cases of colon carcinoma (up to 85%) display chromosomal instability, which manifests as aneuploid and polyploid karyotypes as well as multiple structural chromosomal changes such as allelic losses and mutation of *APCB* and *KRAS*. The remainder of CRC patients (15%) demonstrates microsatellite instability.^[5] Although definitive causes of CRC are unknown, there are a number of factors associated with an increased risk of this disease. In addition to genetic factors, many environmental factors have been attributed as causal factors for CRC; They include diet (*e.g.* high red meat consumption containing saturated fats), obesity, low physical activity, consumption of alcoholic, smoking and inflammatory bowel diseases.^[6] The transition to a more Western diet has been associated with increasing rates of disease.^[7]

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Circulating tumor cells of CRC have been of great interest in cancer research. In blood, their presence appears to be an early marker for recurrence and relapse.^[8,9] There is a correlation between the amount of circulating DNA with stage and response to treatment. It has been observed that the DNA levels in patients with metastatic disease were higher compared to those with non-metastatic diseases.^[10,11]

Mutant *KRAS* is found in CRC and codons 12 and 13 are two hot spots, which account for about 95% of all mutation types, with approximately 80% occurring in codon 12 and 15% in codon 13.^[12] Other mutations in codons 61, 146 and 154 occur less frequently in CRC, accounting for 5% of all mutation type. *KRAS* mutations are preserved throughout the cancer development and can serve as excellent biomarkers for diagnostic testing^[13] and an isoleucine to lysine polymorphism at codon 1307 of the *APCB* gene (I1307K) creating hyper mutable area. Several studies have confirmed the magnitude of risk of CRC conferred by this polymorphism is about 1.8 times than the risk among non carriers.^[13]

Allele specific PCR or Amplification refractory mutation system (ARMS) is accomplished with specially designed primers of defined lengths, which hybridize to the target at the mutation potentially producing mismatches. The premise is that mismatches in the primers, reducing the efficiency in the amplification step.^[14] The direct sequencing has been applied for detection of point mutations which is considered as golden standard method for this type of analysis.^[15]

In this study, amplification of two marker genes and sequenc-

ing method are used for confirming results of the variants present in CRC patients in Sulaimania Province /Kurdistan/Iraq. The current study aims to detect I1307K variant of *APCB* gene and G12S, G12C, G12R, G12D, G12A, G12V, G13D mutations of *KRAS* gene in the blood samples by ARMS-PCR analysis in CRC patients.

2. METHODS

Blood samples were obtained from Hewa Hospital in Sulaimania province Kurdistan-Iraq and samples were transported in cool box to laboratory and tested after up to 2 hours from taking the samples. Two ml of venous blood was collected in EDTA tubes from two groups. 30 unrelated males and females were clinically diagnosed as CRC patients at stage III and IV from different ages between (30-69) years old and 10 samples from healthy people. Extraction of genomic DNA from whole blood was according to.^[16] Finally, the DNA samples were sub- divided into two tubes, one of them for Allele specific mutation for detection of *APCB* gene and the other for Allele specific mutation for detection of *KRAS* gene by (PCR-ARMS).

2.1 PCR amplification of *APCB* gene

Amplification of *APCB* gene required three types of primers, normal primers, common primers and mutant primers (see Table 1), (I1307K) variant of *APCB* gene amplification was done with and without internal control. The DNA region from *APCB* gene was amplified by (allele-specific PCR) using specific oligonucleotide primers, and the PCR amplification was according to primer manufacture.

Table 1. DNA primers (A, B, C)

Primer	Primer's sequence	Amplicon size
(A) <i>APCB</i> Gene ^(1,*)		
F-Mutant type (S3)	5'CTA ATA CCC TGC AAA TAG CAG AAG A3	255
F-Wild type (S1)	5'CTA ATA CCC TGC AAA TAG CAG AAG T3'	255
Reverse primer (S6)	5'TGA GTG GGG TCT CCT GAA CAT A3'	255
(B) <i>KRAS</i> gene ²		
F-Serine (G12S)	5'AAT ATA AAC TTG TGG TAG TTG GAG CG A3'	328
F-Arginine (G12R)	5'AAT ATA AAC TTG TGG TAG TTG GAG CTC3	328
F-Cysteine (G12C)	5'AAT ATA AAC TTG TGG TAG TTG GAG CCT3	328
F-Aspartic acid (G12D)	5'ATA TAA ACT TGT GGT AGT TGG AGC GGA3'	323
F-Alanine (G12A)	5'ATA TAA ACT TGT GGT AGT TGG AGC TTC3	322
F-Valine (G12V)	5'ATA TAA ACT TGT GGT AGT TGG AGC AGT3'	323
F-Aspartic acid (G13D)	5'TAA ACT TGT GGTAGT TGG AGC TGG AGA3'	317
Reverse (S23)	5'ATG CAC AGA GAG TGA ACA TCA TGG AC3	317
(C) β -Actin Gene ³		
F-Internal Control	5'TAT GTG GGC GAC GAG GCC CA3'	353
R-Internal Control	5'GCA TAC CCC TCG TAG ATG GG3'	353

Note. (A) *APCB* primer (17); (B) *KRAS* primer (our design); (C) β - actin primer (our design).

2.2 Gel electrophoresis for *APCB* gene detection

PCR products were analyzed by gel electrophoresis on 1% agarose gel in 1X TBE buffer at 90 V, 100 mA, 9 W for one hour. 10 μ L of PCR products both (patient & healthy) samples was loaded in to the agarose gel directly, and 5 μ L of 100 bp DNA ladder was loaded. The DNA was stained by ethidium bromide and the PCR product was visualized under UV light.

2.3 PCR amplification of *KRAS* gene with and without internal control

Purified genomic DNA was examined by the amplification refractory mutation system assay which is developed to detect specific point mutations in the *KRAS* oncogene. The 3'-terminal base (see Table 1, bases are labeled red) of each of 7 oligonucleotide primers was complementary to one of the common mutations of codon 12 and 13 of *KRAS* gene (12Ser, 12Arg, 12Cys, 12Asp, 12Ala, 12Val, 13Asp). Sample DNA is amplified with eight separate primer sets (one for the wild-type sequence and one for each of seven point mutations) with internal control (see Table 1).

2.4 Gel electrophoresis for *KRAS* gene detection

PCR products were analyzed by gel electrophoresis on 1% agarose gel in 1X TBE buffer at 90 V, 100 mA, 9 W for an hour. 10 μ L of PCR products of patients was loaded into the agarose gel directly, and 5 μ L of 100 bp DNA ladder was loaded. The DNA was stained by ethidium bromide and the PCR product was visualized under UV light. The presence of (328, 323, 322, 317) bp fragment indicated the positive result, on other hand, the presence of (353) bp fragment indicated the internal control band. Also both were estimated according to the migration pattern of 100 bp DNA ladder.

2.5 Amplification of *APCB* and *KRAS* gene in CRC patients for sequencing

The amplification of two genes and the agarose gel electrophoresis of 2 patient samples showed 406 bp fragment for amplified product of *APCB* which contain the specific variant in Figure 4. The other band 236 bp fragment which indicated to amplified product of *KRAS* which contain the mutations at codon 12 and 13 in Figure 5. After PCR purification of both samples, they were provided for DNA Sequencing. Before sequencing, the PCR products were purified by PCR purification Kit, both methods (purification and sequencing) were done in the "Genome Center" laboratory in Koya University. Two pairs of primer used one for *APCB* gene (F-*APCB* 5' AGG CTG CCA CTT GCA AAG TTT 3', R-*APCB*, 5' TGA GTG GGG TCT CCT GAA CAT A3, sequencing of 406 bp) (our design) and other for *KRAS* gene (F-*KRAS*, 5' GTG TGA CAT GTT CTA ATA TAG TCA 3', R-*KRAS*, 5'GAA TGG

TCC TGC ACC AGT AA3) for sequencing of 236 b.p.^[18] The forward and reverse sequences were aligned by using Bio-Edit program. The complete sequence was BLAST on NCBI online website. APE software was used for alignment of the sequences with genomic sequences of both genes in the Gene Bank Data Base.

2.6 Sequencing and alignment of (I1307k) *APCB* gene and of 7 common mutation codons of *KRAS* gene variants

A total of two samples out of 30 PCR samples were randomly submitted to cycle sequencing reaction in Genome Center Laboratory at Koya University. These two samples were clinically diagnosed with CRC. Sequence region of *APCB* gene on chromosome 5 and their flanking regions and sequence region of *KRAS* gene on codons 12 (G12S, G12C, G12A, G12R, G12D, G12V) and 13 (G13D) with their flanking regions recovered using the nucleotide sequence search program on the Entrez Browser Website provided by the National Center for Biotechnology Information (NCBI).

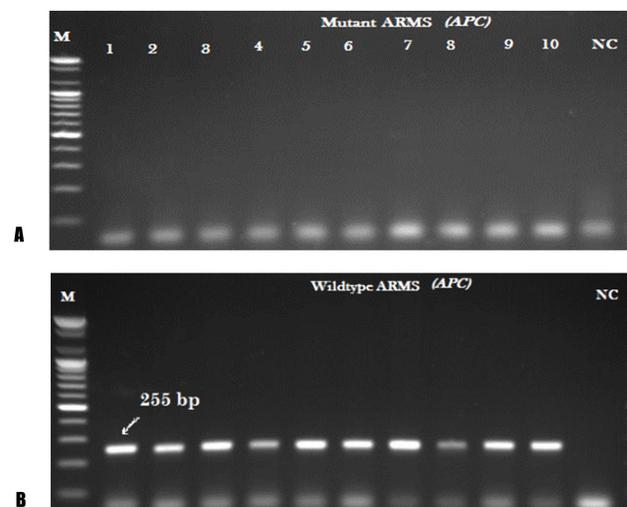


Figure 1. A) Gel electrophoresis for ARMS PCR product from patient samples. M: DNA ladder marker (2 kb); NC: negative control, Lane 1-10 patient samples represent negative specific PCR products (1307K) by using Mutant Primer (lysine ARMS primer). B) Gel electrophoresis for ARMS-PCR product from healthy samples; M: DNA ladder marker (2 kb); NC: negative control, all healthy samples represent positive wild type PCR products (255 bp) by using Wild type primer (isoleucine).

3. RESULTS AND DISCUSSION

Totally 30 samples were clinically diagnosed as CRC patients in stage III and VI, and their ages were between (30-69) years old. In addition, 10 apparently healthy subjects, 5 male and 5 female from ranging ages (23-50) years old were included.

Genomic DNA is extracted from blood samples and applied for allelic specific PCR assay.

3.1 Amplification of APCB gene

The amplification of (I1307K) variant of *APCB* gene and the agarose gel electrophoresis of 30 patient samples showed no band for this polymorphism by using Mutant ARMS primer (see Figure 1A), while the amplification of wild type (I1307K) variant of *APCB* gene and the agarose gel electrophoresis of 10 healthy samples showed band of 255 bp by using wildtype ARMS primer Figure 1B. This band was found in 100% of healthy samples, but not in patient samples. Results obtained in the present work, where healthy samples were used, wild type primer showed (255 bp) band as an indicate to positive results, in agreement with^[17] in United Kingdom, who used the same wild type primer to healthy groups and showed Wild type PCR product at (255 bp).

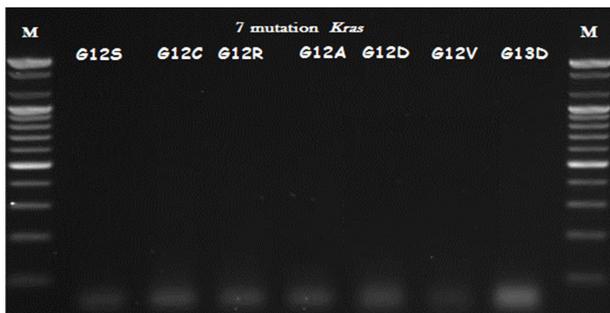


Figure 2. Gel electrophoresis for ARMS-PCR products from patient samples. M: DNA ladder marker (2 kb); each patient sample represent negative PCR products for all six mutation at codon 12(G12S, G12C, G12R, G12A, G12D, G12V) and one at codon (G13D) of *KRAS* gene by using specific ARMS primers.

3.2 Amplification of KRAS gene in CRC patients with (7 mutant ARMS)

Depending on ARMS technique, the amplification of *KRAS* gene for 7 mutations at codons 12 and 13 by using specific primer for each unique mutation and the agarose gel electrophoresis of 30 patient samples showed no band for these mutations by using 7 Mutant ARMS primer (see Figure 2). The results which obtained from each patient was 100% negative (e.g. wildtype) of all mutation types. The results were similar to^[17] in Egypt, who observed negative results (Wildtype) *KRAS* in both codon 12 and 13.

3.3 Amplification of APCB and KRAS gene in CRC patients with internal control

The amplification of *APCB* and *KRAS* were done in the present of the primers for *actβ* as internal control which gives an amplicon with 353 bp in size which appears to be found in all patients and healthy samples (see Figure 3).

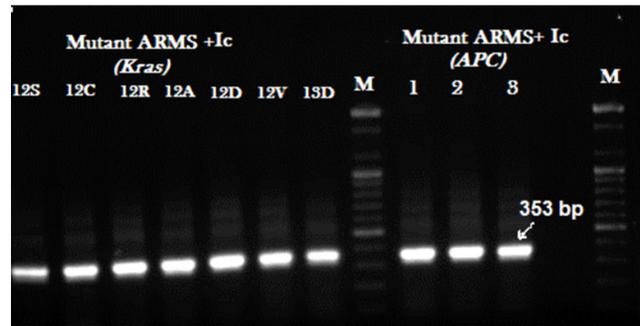


Figure 3. Gel electrophoretic pattern of PCR amplification of *APCB* (I1307K) variant and (7 mutation codon 12 and 13) of *KRAS* gene in patients with *Ic*. M: DNA ladder marker (2 kb) Lanes 1-3 represent negative PCR amplification products of *APCB* variant and positive of β -actin (353 bp). Lanes (12S, 12C, 12R, 12A, 12D, 12V, 13D) all mutation type of *KRAS* gene represent negative PCR products of *KRAS* mutation and positive of β -actin (353 bp).

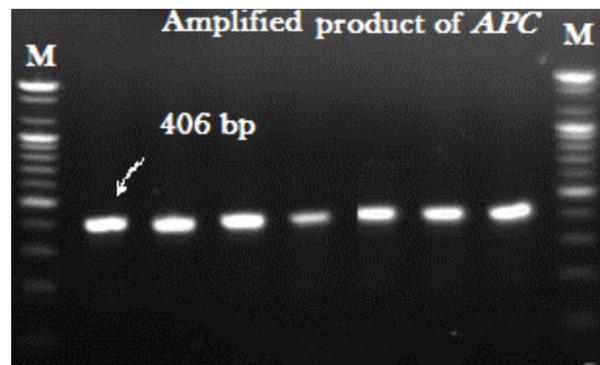


Figure 4. Gel electrophoresis pattern of PCR amplification of *APCB* gene for Sequencing. M: DNA ladder marker (2 kb); All Lanes represent PCR amplification products of *APCB* gene (406 bp) fragment.

The selection of (I1307K) polymorphism in the present work is due to a number of reasons: A genetic variant of *APCB*, (I1307K) which causes to frame-shift mutations resulting in subsequent somatic inactivation of the *APCB* gene product.^[20,21] Therefore, it represents further alterations which promote tumorigenesis and many reports confirmed that carrier of this specific polymorphism has a relative risk of 1.5-2.0 for CRC than non-carrier.^[22] Also it has been associated with the increased adenomatous polyp formation which may also be a cause in an increased risk of transition from adenoma to invasive cancer.^[23] Finally, this allele is considered as the ancestor of modern I1307K alleles existed 87.9-118 generations ago (2, 200-2, 950 years ago)^[24] by evolution, whether this allele is carried in our people or not?

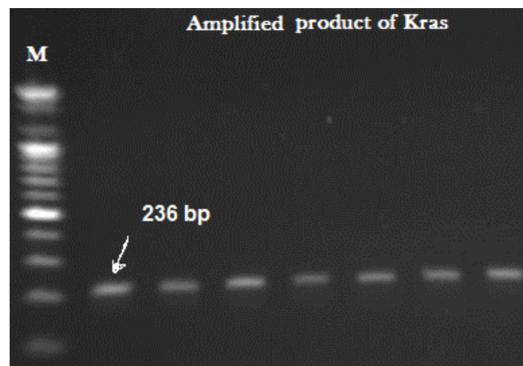


Figure 5. Gel electrophoretic pattern of PCR amplification of *KRAS* gene for Sequencing. M: DNA ladder marker (2kb). All Lanes represent PCR amplification products of *KRAS* gene (236 bp) fragment.

The current study shows that out of the total 30 blood samples, the results were (100%) negative (Wild type) for specific polymorphism of *APCB* gene. This result is matched with the result by^[25] who obtained (98%) wild type of this variant. Another study by^[26] has demonstrated that prevalence was higher among Ashkenazi Jews 11.2% than among non-Ashkenazi Jews 2.7% and only 3% of Arabs population carry this polymorphism. Also it was reported that this mutation cannot be found in any Arabs or non-Ashkenazi Jews other than Jews from Yemen in which the mutation was reported extremely high.

Interpretation of allele-specific PCR assays is based on the amplification of a PCR product in the presence of the respective mutation. The absent of the PCR product in the reaction is interpreted as wild-type sample.^[14] In the current study, a lack of amplification can be due to the presence of PCR inhibitors. Therefore, integration of an internal control reaction β -actin is essential to exclude false-negative results. In order to minimize competition of the internal control reaction with the target gene amplification, fewer internal control primers compared with target-specific primers were used in the reaction.

The present work demonstrates that out of 30 patients who have negative results (*APCB* wild type) for this variant. Although this variant is considered as conserved allele which is shared among Jews and Arabs and because the allele age of I1307K indicates that the most recent ancestor of I1307K existed sometime between (947 BC and 195BC).^[23] It was observed that may be by genetic exchange this allele has low prevalence in almost Arabic world. It was also appeared rare variant in Sulaimania province.

It was clear that the importance of activating mutations in the *KRAS* gene in relation to carcinogenesis and their importance as diagnostic biomarkers provides clues regarding human

cancer patients and prognosis indicating potential therapeutic approaches.^[27] The *KRAS* mutations at codon 12 and codon 13 have been widely studied in CRCs in Western countries and the prevalence of *KRAS* mutation representing 47% in USA, 35% in Brazil, 34% in Spain and 45.5% in Slovenia.^[28] Conversely, there are few data on *KRAS* mutation rate and spectrum in CRCs from Middle East countries, such as Saudi Arabia and Jordan 28% and 44% respectively^[29] and based on a study done in Tehran, the *KRAS* mutation rate was 37.4%.^[30]

These Eastern countries share certain cultural background and environmental exposures. Thus, ethnicity and geographical differences may also have role in distribution of prevalence *KRAS* mutation rate around the world. It was appeared in low rate in some of Asian countries compared with European countries.

In this research our finding show, all mutation types in codon 12 and 13 were negative (Wild type) *KRAS*. It was similar to an Egyptian study reported that mutations of the *KRAS* is uncommon in Egyptian CRC cases in contrast to Western cases.^[31] Therefore, these mutations in CRC patients of this study were observed uncommon or may be rare in Sulaimania province. Several reasons were explained in addition to genetic factors. Accumulating evidence suggests that environmental factors such as diet could be involved in CRC progression and could induce specific *KRAS* mutations. Diet-related carcinogens can induce *KRAS* mutations^[6] and dietary components were shown to influence the rate and spectrum of *KRAS* mutations in CRC.^[7] Dietary factors may also affect clonal selection by modifying the growth of tumors harboring specific *KRAS* mutations. The absences of I1307K mutation of *APCB* gene and 7 common mutation of *KRAS* gene is not surprising because the specific variant of *APCB* has been reported rare variant according to Arabs population and the limitation studies about this polymorphism in Middle East countries. Although seven common mutations of *KRAS* gene have been reported in high rate of mutation in wisteria countries but noticed in low rate in Middle East countries such as Saudi Arabia and Egypt. According to the results obtained from different techniques, in the present study in Sulaimania province. These eight variants of both genes were absent in our CRC patients. The geographic distribution plays an important role in the presence and absences of rate mutation of both genes. Thus, geographic area causes the possibility of more variants to occur in Kurdish population which differs from neighboring countries and his research is considered to be the first study on using ARMS assay for detection of specific mutations in *KRAS* ontogeny and *APCB* tumor suppressor gene for CRC in Kurdistan region of Iraq,

3.4 Alignment of APCB gene

The forward and reverse sequences were aligned by using Bio Edit program; the complete sequences of both samples

were Blast on (NCBI) online Website and its genotype was determined.

```

Query 1  CATTATCATCTTTGTCATCAGCTGAAGATGAAATAGGATGTAATCAGACGACACAGGAAG 60
|||||
Sbjct 151901
CATTATCATCTTTGTCATCAGCTGAAGATGAAATAGGATGTAATCAGACGACACAGGAAG 151960

Query 61
CAGATTCTGCTAATACCCTGCAAATAGCAGAAATAAAAGAAAAGATTGGAAGTGGTTCAG 120
|||||
Sbjct 151961
CAGATTCTGCTAATACCCTGCAAATAGCAGAAATAAAAGAAAAGATTGGAAGTGGTTCAG 152020

Query 121
CTGAAGATCCTGTGAGCGAAGTTCCAGCAGTGTACAGCACCCCTAGAACCAAATCCAGCA 180
|||||
Sbjct 152021
CTGAAGATCCTGTGAGCGAAGTTCCAGCAGTGTACAGCACCCCTAGAACCAAATCCAGCA 152080

Query 181  GACTGCAGGGTTCTAGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTT 240
|||||
Sbjct 152081
GACTGCAGGGTTCTAGTTTATCTTCAGAATCAGCCAGGCACAAAGCTGTTGAATTTTCTT 152140

Query 241  CAGGAGCGAA 250
|||||
Sbjct 152141 CAGGAGCGAA 152150

```

↓ Wild type (I1307K) variant

↖ Wild type (I1307K) variant

3.5 Alignment of KRAS gene

The forward and reverse sequences were aligned by using Bio Edit program; the complete sequence of both samples was Blast on NCBI online Website and its genotype was determined. Based on the obtained results from ARMS-PCR technique, to avoid false negative results and confirmation from accurate results. In this study, DNA sequencing method

is used showing all mutation in template, if present. Therefore, our two sequence alignment of Sulaimania CRC samples with genotype prototype sequence selected from the Gene Bank. CRC is one of the commonest malignant tumors worldwide. The incidence rate ranks third in all tumors; it is almost diagnosis by histopathology using TNM classification.^[32]

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Query 1  GTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAGGCCTGCTGAAAA 60
|||||
Sbjct 10478 GTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAGGCCTGCTGAAAA 10537

Query 61  TGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGA 120
|||||
Sbjct 10538
TGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGA 10597

Query 121 TACAGCTAATTCAGAATCATTGTGGACGAATATGATCCAACAATAGAGGTAAATCTTG 180
|||||
Sbjct 10598 TACAGCTAATTCAGAATCATTGTGGACGAATATGATCCAACAATAGAGGTAAATCTTG
10657

Query 181 TTTTAATATGCATATTACTGGTGCAGGACCATTC 214
|||||
Sbjct 10658 TTTTAATATGCATATTACTGGTGCAGGACCATTC 10691

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↙ ↘ Wildtype *Kras*

↖ ↗ Wildtype *Kras*

4. CONCLUSIONS

The ultimate goal of this investigation is to determine seven common mutations in *KRAS* gene and I1307K in *APCB* gene in colorectal carcinoma by using ARMS-PCR technique. The eight variants of both genes are absent in our CRC patients. Thus, the geographic distribution might play an important

role in the presence and absences of the rate of mutations in both genes.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare that there is no conflict of interest statement.

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