RESEARCH ARTICLE

Editorial Process: Submission:10/16/2019 Acceptance:11/17/2023

Mutation Analysis of *KRAS* and *BRAF* in Iranian Colorectal Cancer patients: A Novel Variant in Exon 15 of BRAF

Bita Hassani[1], Rasoul Alizadeh[1], Mansoureh Akouchekian[1]*, Fahimeh Safarnezhad Tameshkel[2], Mohammad Hadi Karbalaie Niya[3]

Abstract

Background: Mitogen-Activated Protein Kinase (MAPK) pathway and its downstream signaling pathways, play an important role in intracellular signaling. Mutations in KRAS (activating mutation) and *BRAF* proto-oncogenes are identified as key finding of colorectal cancer. The aim of this study was to examine mutation analysis of *KRAS* and *BRAF* in Iranian Colorectal cancer patients. **Methods:** We used fifty archived formalin fixed paraffin-embedded (FFPE) blocks of Iranian colorectal cancer patients. DNA was extracted from FFPE blocks for PCR assay. The quality of PCR products was determined using horizontal electrophoresis. Then, sequencing and analysis of the sequencing results were performed to investigate variation status in the sequences. **Results:** *KRAS* exons and *BRAF* genes exon 15 in 50 CRC patients were analyzed, among the 19 mutant KRAS samples, 18 (36%) patients had a single base substitution (synonymous mutation) in exon 5, p. Arg161Arg (c.483G>A) and 1 (2%) patient in exon 2 (codon 12), p. Gly12Cys (c.34G>T). Also, we observed two mutations p. Val600Glu (c.1799 T>A) and p. Ser616Thr (c.1846T>A) in exon 15 of *BRAF* gene. **Conclusions:** We found a novel variant in *BRAF* gene. The p. Ser616Thr (c.1846T>A) mutation was not previously reported and we conclude that other new mutations can be identified in *KRAS* and *BRAF* which may lead to colorectal cancer.

Keywords: Colorectal neoplasms- *KRAS*- *BRAF*- mutation analysis- proto-oncogene

Asian Pac J Cancer Prev, **24 (11)**, 3665-3671

Introduction

Colorectal cancer (CRC) is a heterogeneous malignancy and serious health issue in both of men and women. There will be an estimated approximately 1 million new CRC cases and 551,269 deaths, anniversary (Siegel et al., 2017, Bray et al., 2018). CRC is the fourth common cause of cancer mortality worldwide that almost 20% of them involved by metastases (Zhao et al., 2017). However, in the past two decades due to broader screening procedures and surgical therapy, incidence of CRC has been reduced (El-Shami et al., 2015). Approximately in the 70% of CRC cases cancer development initiated from colon and in 30% rectum is the first part (Wolpin et al., 2007). CRC is a multifactorial disease in which lifestyle, diet habit, gender, alcohol consumption, smoking, low physical activity, age, genetic and environmental factors contribute to disease pathogenesis (Asghari-Jafarabadi et al., 2009). Also, the diverse types of mutations identified in CRC patients such as *APC, KRAS, p53* genes and Deleted in Colorectal Cancer (DCC) in 18q12.2, microsatellite instability, germline mutation (mismatch repair genes, MLH1- MSH2), hypermethylation (MLH1) and chromosome instability (Jayasekara et al., 2017, Clarke and Kopetz, 2015). Mitogen-Activated Protein Kinase (MAPK) pathway and its downstream signaling pathway such as RAS-RAF-MEK-ERK plays an important role in intracellular signaling. MAPK pathway involved in regulation of proliferation, mitosis and cell survival which normally KRAS stimulate cell cycle progression (Jayasekara et al., 2017, Larki et al., 2017, Dhomen and Marais, 2007). KRAS and BRAF are risk gene of CRC that approximately 36-40% of CRC patients harbor a mutation in KRAS and 8-15% in BRAF (McCoy et al., 1983, Schubbert et al., 2007). Although, KRAS has an approximately 40% -45% mutations in codons 12 and 13 of exon 2, rare mutations at codons 61 and 146 detected (Harada et al., 2007, Kranenburg, 2005, Fearon and Vogelstein, 1990). Overall BRAF mutation is about 5% to 10% of cases (Harada et al., 2007, Larki et al., 2017). The majority of BRAF mutations (more than 90%) are in the hotspot of exon 15 resulting in the V600E substitution (GTG to GAG) (Larki et al., 2017, Dhomen and Marais, 2007, Harada et al., 2007). *KRAS* mutation frequency is

[1]*Department of Medical Genetics and Molecular Biology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran.* [2]*Gastrointestinal and Liver Diseases Research Center, Firoozgar Hospital, Iran University of Medical Sciences, Tehran, Iran.* [3]*Institute of Immunology and Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran. *For Correspondence: akouchekian.m@iums.ac.ir*

different in any stage of CRC. Its mutation rates in late adenoma stage is more than the rest of other stages. The chromosome 17p deletion in which *p53* located there, is more common than *KRAS* mutation in the carcinogenesis (Fearon and Vogelstein, 1990). Detection of cancerous cells mutations have significant impact on efficiency of therapy. CRC complications involved by nearly 140 genes and the complexity of treatment increased by each mutation (Maresso et al., 2015). For instance, one of treatment strategies is based on implication of monoclonal antibody (Cetuximab) that inhibit EGFR pathway. Via a *KRAS* mutation, Cetuximab could not inhibit this signaling pathway (Van Cutsem et al., 2011, Wolpin et al., 2007). The aim of the present study was to evaluate the mutation rate of *KRAS* and *BRAF* genes in CRC patients in order to better defining the correlation of these mutations with worsen complications and use these data for further treatment strategies and epidemiological studies.

Materials and Methods

Patients and tissue samples

A total of fifty formalin fixed paraffin-embedded (FFPE) blocks of CRC cases were obtained from pathology repository of Firoozgar Hospital, Tehran, Iran that affiliated to Iran University of Medical Sciences, Tehran, Iran, from 2010 to 2015. The presence of malignant cancer in the patients confirmed by the pathologist and their characteristics are summarized in Table 1. Consent forms were signed by the all patients. This research is reviewed and approved by the constituted Ethics Committee of Iran University of Medical Sciences (Tehran, Iran, IR.IUMS.FMD.REC.1394.26544).

DNA extraction

Genomic DNA was extracted from fifty 10-µm-thick FFPE tissue sections using the DNA FFPE Tissue Kit (NucleoSpin®Tissuekit, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were assessed using spectrophotometry and DNA with an appropriate optical-density ratio was used.

PCR amplification

Altogether, the sequences of exons 2, 3, 4, 5 and 6 of *KRAS* gene and exon 15 of *BRAF* gene were analyzed by polymerase chain reaction. The PCR reaction took place in a volume of 25 µl, including 12.5 µl of Master mix (Ampliqon, Denmark), 1 µl of each primer, 6-7 µl of genomic DNA and 3.5-4.5 µl of distilled water. DNA was amplified according to the following program: an initial denaturation cycle of 95°C for 5 min; 40 cycles of denaturation (95°C for 60 s), annealing (60 s at 57°C for KRAS exon 2, 4, 6 or at 59°C for KRAS exon 5 or at 55°C for KRAS exon 3 or at 57°C for BRAF exon 15), and elongation (72°C for 1 min); and a final extension cycle at 72°C for 10 min, using thermal cycler machine (Bio-Rad, USA). The accuracy of the target amplification was analyzed by electrophoresis of PCR products on 1% agarose gel. The genomic DNA was amplified using the primers listed in Table 2.

Direct Sequencing

The purified samples by High Pure PCR Product Purification Kit (Roche Diagnostic GmbH, Mannheim, Germany) together with 20 µl of related primers were used for sequencing via ABI 3700 sequencer (PE Biosystems, Foster City, CA, USA). Analyses of the DNA sequences were performed by the Sequence Scanner Software and Sequencer software version 5.

Alterations were determined by alignment of sequencing results against a reference sequence database. Codon number of the mutant gene and its changed amino acid sequence was determined based on GenBank entry NM_004985, NP_004976.2 for KRAS and NM_001354609.1, NP_001341538.1 for BRAF as references.

Computational analysis

Pathological impact of found variants was predicted by tools consisting of PolyPhen-2 (Adzhubei et al., 2010), Sorting Intolerant from Tolerant (SIFT) (Kumar et al., 2009), Project Hope (http://www.cmbi.ru.nl/hope/method/) (Venselaar et al., 2010), CADD (Combined Annotation Dependent Depletion) (Kircher et al., 2014), provean (Choi and Chan, 2015), FATHMM (Shihab et al., 2013) and mutation taster (Schwarz et al., 2014). The FASTA sequence of BRAF (human) (Uniprot: P15056) was used as the reference for generated 3D protein structure models in SWISS-MODEL (Waterhouse et al., 2018). The protein structure was energy minimized by Swiss-Pdb viewer and then the validation of generated protein structural model performed by Ramachandran plot analysis, 100% of the residues in homology model were observed in allowed and favored regions. In this study, peptide designing, Molecular graphics and analyses of BRAF (Ser616Thr) were performed with the UCSF Chimera version 1.13.1.

3D structure of mutant ARSB protein (right) and the wild type structure (left) in ribbon and surface presentation made by Chimera software, with two altered amino acids (Arg and Ala) which is indicated in cyan color and wild type form of them (His and Pro).

Results

Amplification and direct sequencing of the exons 2, 3, 4, 5 and 6 of KRAS gene and exon 15 of *BRAF* gene in fifty Iranian patients with CRC were successfully carried out. For the KRAS exons 3, 4 and 6, obtained sequences were compared to the reference sequences. Codon number of the mutant gene and its changed amino acid sequence was determined based on GenBank entry NM_004985, NP_004976.2 for KRAS and NM_001354609.1, NP_001341538.1 for BRAF as references. Totally, of 50 samples, 19 patients (38%) were detected with mutation in KRAS gene and two patients (4%) in exon 15 of BRAF gene. Moreover, 47.36% (9/19) and 52.63 % (10/19) KRAS mutations were in male and female patient, respectively. While just only 15.78 % (3/19) identified in patients under 50 years of age. Among the 19 mutant *KRAS* samples, 18 (36%) patients had a single base substitution (synonymous mutation) in

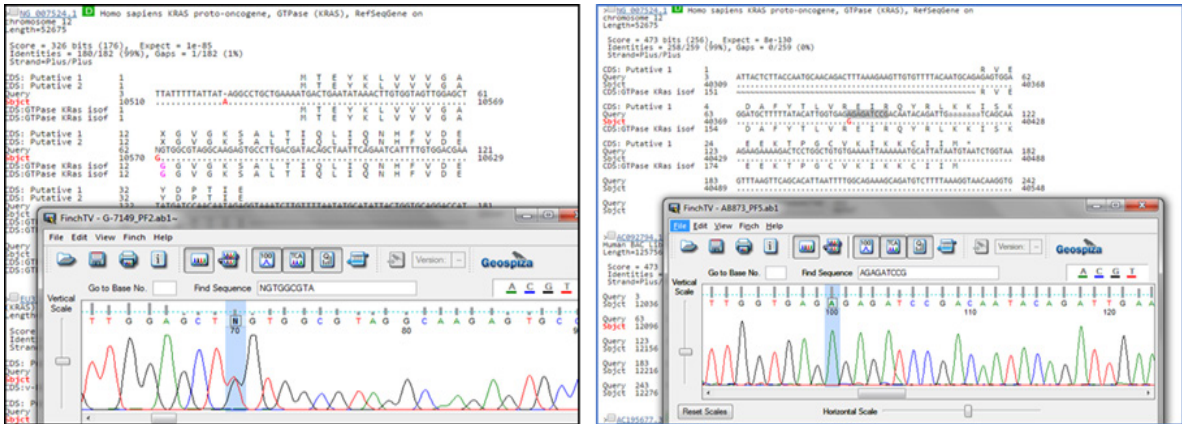

Figure 1. A DNA Sequence Electropherogram of *KRAS* Gene Mutation (forward direction): A: c.34G>T(p. G12C) hetero of exon2. B: c.483G>A (p. R161R) homo of exon 5.

exon 5, p. Arg161Arg (c.483G>A) and 1 (2%) patient in exon 2 (codon 12), p. Gly12Cys (c.34G>T). Also, we observed two mutations p. Val600Glu (c.1799 T>A) and p. Ser616Thr (c.1846T>A) in exon 15 of *BRAF* gene. The frequency of *KRAS* and *BRAF* mutations according to clinical and pathological features summarized in Table 3. Representative electropherograms of *KRAS* and *BRAF* mutations are shown in Figures 1 and 2, respectively. Sequence analysis identified a novel heterozygous variant, c.1846T>A in *BRAF* that caused the amino acid substitution of serine with threonine (S616T). Via bioinformatics data, we found that p. Ser616Thr (c.1846T>A) mutation was not previously reported. Frist of all, the missense mutation effect was assessed using mutation taster (Schwarz et al., 2014) (http://www.mutationtaster.org/) which predict that this mutation (c.1846T>A) was disease-causing. So, we ranked the variant by applying the Combined Annotation Dependent Depletion (CADD) tool v1.3 (Rentzsch et al., 2019) (http://cadd.gs.washington.edu/). The variant (c.1846T>A) with the scaled PHRED CADD score of >28 which is considered damaging. On the other hand, novel variant were predicted damaging by sift (Kumar et al., 2009) (http://sift.jcvi.org/), provean (Choi and Chan, 2015) (http://provean.jcvi.org/index.php) and FATHMM (Shihab et al., 2013) (http://fathmm.biocompute.org.uk/). According to, American College of Medical Genetics and Genomics (ACMG) guidelines the novel variant classified as a likely pathogenic mutation (Richards et al., 2015). When we searched for the effect of novel damaging variant on protein stability by MUpro (http://mupro.proteomics.ics.uci.edu/), we observed that this mutation decrease the protein stability. As well as, through in silico analysis,

Table 1. Demographical and Pathological Characteristics of 50 Iranian CRC Patients

Clinical and pathological characteristics		Total (n= 50) N (%)
Gender	Male	28 (56)
	Female	22 (44)
Age (y)	≤50	15 (30)
	>50	35 (70)
Location	Colon	21 (42)
	Rectum	12 (24)
	Cecum	8 (16)
	Ileum	2 (4)
	Sigmoid	7 (14)
pT (Depth of invasion)	NO	2 (4)
	p Tis	1 (2)
	p T1	3 (6)
	p T2	8 (16)
	p T3	26 (52)
	p T4	10 (20)
Tumor type	Mucinous adenocarcinoma	15 (30)
	Non-mucinous adenocarcinoma	29 (58)
	Missed data	6 (12)
Tumor differentiation	Well	28 (56)
	Moderate	15 (30)
	Poor	1 (2)
	Un-differentiated	5 (10)
	Missed data	1 (2)
Pathological Grade	Low	21 (42)
	High	22 (44)
	Missed data	7 (14)
Lymph nodes involvement	Involved	12 (24)
	Uninvolved	38 (76)

Table 2. Primers Used for FLG mutation Analysis

Gene (Exon region)	Primer pairs 5'→ 3'	Size
Kras (exon 2)	F:5'GTGTGACATGTTCTAATATAGTCA3'	213bp
	R: 5'GAATGGTCCTGCACCAGTAA3'	
Kras (exon 3)	F:5'TCAAGTCCTTTGCCCATTTT3'	374bp
	R: 5'TGCATGGCATTAGCAAAGAC3'	
Kras (exon 4)	F:5'TTGTGGACAGGTTTTGAAAGA3'	379bp
	R: 5'AGAAGCAATGCCCTCTCAAG3'	
Kras (exon 5)	F:5'CTCAAGCTCATAATCTCAAACTTCT3'	305bp
	R: 5'GTAGTTCTAAAGTGGTTGCCACC3'	
Kras (exon 6)	F:5'GACAAAACACCTATGCGGATGA3'	429bp
	R: 5'GCTAACAGTCTGCATGGAGCA3'	
BRAF (exon15)	F:5'TCATAATGCTTGCTCTGATAGGA3'	223bp
	R: 5'GGCCAAAAATTTAATCAGTGGA3'	

Table 3. Frequency of the Observed Mutations in *KRAS* and *BRAF* Genes in Iranian CRC Patients

	All mutations	
	KRAS	BRAF
Clinical and Pathological feature	19 (38%)	2 (4%)
	p. R161R p. G12C (c.483G>A) (c.34G>T)	p. V600E p. S616T (c.1799 T>A) (c.1846T>A)
Gender		
Male	8 (16%)	1 (2%)
Female	10 (20%)	1 (2%)
Age (y)		
≤50	3 (6%)	1 (2%)
>50	15(30%)	1 (2%)
Location		
Colon	6 (12%)	colon
Rectum	6 (12%)	colon
Cecum	4 (8%)	
Ileum	-	
Sigmoid	2 (4%)	Sigmoid
pT (Depth of invasion)		
p Tis	-	
p T1	1 (2%)	
p T2	3 (6%)	
p T3	8 (16%)	pT3 pT3
p T4	6	
Tumor type Mucinous adenocarcinoma		
Nonmucinous adenocarcinoma	8 (16%)	Mucinous Non-mucinous
Single ring cell adenocarcinoma	8 (16%)	Nonmucinous
Miss	2 (2%)	
Tumor differentiation		
Well Differentiated	11 (22%)	Well
Moderate Differentiated	5 (10%)	Moderate
Poor differentiated	1 (2%)	
Undifferentiated	1 (2%)	Undifferentiated
Pathological Grade		
Low grade	7 (14%)	
High grade	10 (20%)	
Lymph nodes involvement		
Involved	3 (6%)	Involved
Uninvolved	15 (30%)	Uninvolved

it revealed that substitution of Serine with Threonine at the position of 616(c.1846T>A) changed the amino acid interactions. This replacement lost previous interaction with Asparagine 580 (Figure 3). According to HOPE reports this variant introduced an amino acid with different properties, which can disturb this domain and abolish its function. The family member (patient's daughter) analysis demonstrated the novel variant has not been transmitted to his daughter (Figure 4). Interestingly, mutations in *KRAS* and *BRAF* were not observed in the same cases. We also analyzed Clinical and pathological characteristics on patient's sex and age, tumor type, location, depth of invasion pathological grade, tumor differentiation and lymph nodes involvement (Table 3).

Discussion

Recently, colorectal cancer increased dramatically worldwide which account for 17% of all new cancer cases and as well as 17% of all cancer deaths (Siegel et al., 2017). CRC is one of the most crucial public health problems in Iranian population, because of considerable increasing incidence of CRC over the past years (Asghari-Jafarabadi et al., 2009). Various individual and environmental factors are involved in the development of CRC. Of individual factors, genetically differences in order to ethnical and mutational status in different genes such as KRAS and BRAF are more common in the CRC development (Nistal et al., 2015). In the present study, we conducted genetic alterations assessment in *KRAS* and *BRAF* genes in CRC patients. CRC is a complex disease that has two main contributors to the pathogenesis of this disease. Additionally, specific genetic alterations are cause approximately 10% of all colon cancers (Armaghany et al., 2012). Our study results showed there were 38% *KRAS* and 4% *BRAF* mutants. It shows the higher mutational risk of Iranian CRC population. In molecular targeted therapies determination of the tumorous cells status is a key step. For example, in CRC, patients with *KRAS* mutations have been found to be resistant to treatment with anti-EGFR antibodies. Our study failed to assess the chemotropic drugs that used, but twenty-one (42%) isolates were identified as mutant. Furthermore, of 19 *KRAS* mutant, 18 mutants were detected in exon 5 and

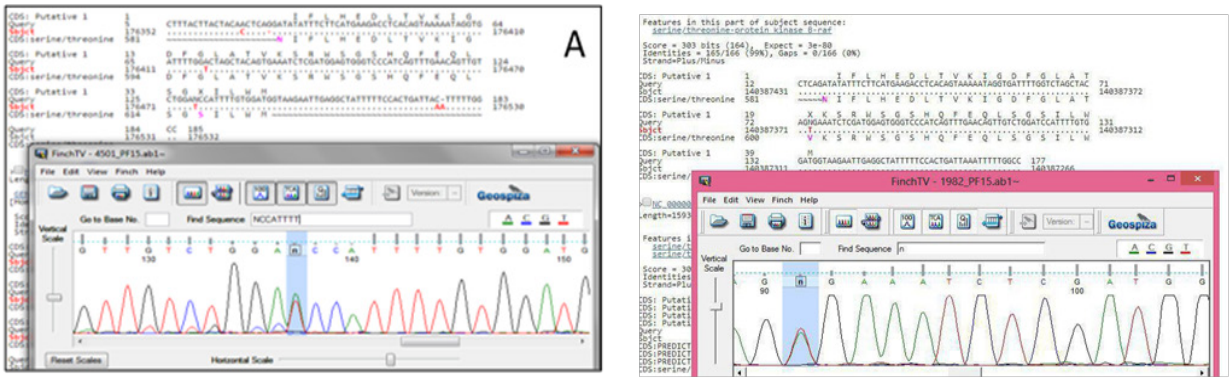

Figure 2. A DNA Sequence Electropherogram of *BRAF* Gene Mutation (forward direction): A: p. S616T (c.1846T>A) hetero of exon15. B: p. V600E (c.1799 T>A) of exon 15

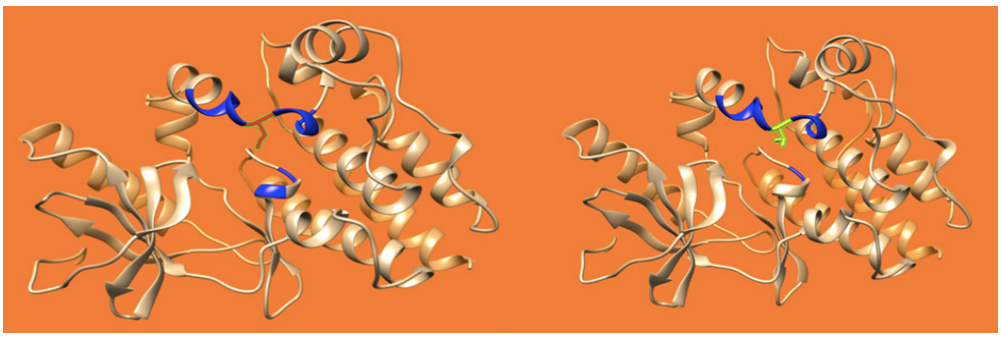

Figure 3. 3D Structure of Mutant *BRAF* Protein (right) and the wild type structure (left) in ribbon and surface presentation made by Chimera software, with an altered amino acids (Thr) which is indicated in green color and wild type form of them (Ser).

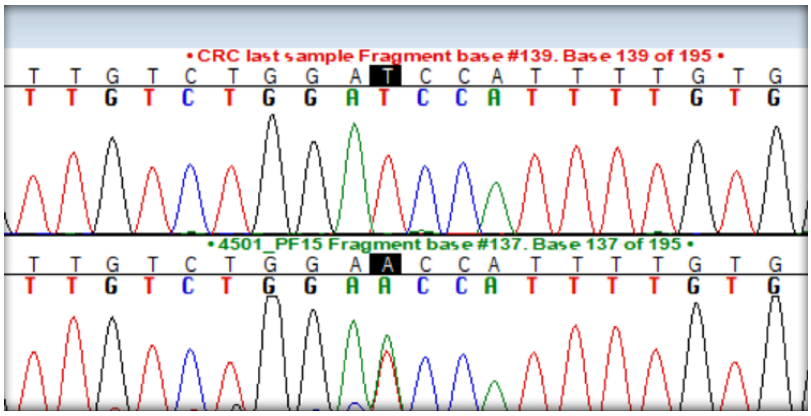

Figure 4. DNA Sequence Electropherogram of *BRAF* Gene

only one mutant (c.34G>T, p. G12C) observed in exon 2 (codon 12). According to the study conducted in Iran which found *KRAS* mutation rate in CRC is 33.6 that was similar to our findings (Koochak et al., 2016). On the other hand, the *KRAS* mutation rate in the European countries, Japan and China, the Middle East has been reported to be 36, 38, 24 percent, respectively (Koochak et al., 2016). The *BRAF* mutation rate is less than 10% in which 80% of them occurred in exon 15 (De Roock et al., 2009). As well, two mutations in exon 15 of *BRAF* were observed in men older than 50 that including one novel variant (p. Ser616Thr (c.1846T>A). This novel variant was not observed in that patient's daughter. The normal allele or the novel variant probably has occurred somatically that daughter. This data could be showed the Iranian CRC patients genetic diversity from other nations and or to be the probable cause of increasing number of CRC cases in Iran. The most mutations in codon 12 for *KRAS* oncogene, are Gly to Asp, Gly to Val and Gly to Cys (Dobre et al., 2015, Faghani et al., 2012, Li et al., 2010). In our study, missense mutation c.34G>T, which causes the Gly to be replaced with Cys was found. While in codon 13 we did not find any genetic changes. In addition, we detected synonymous mutation c.34G>T (p. G12C) in 18 CRC patients which the frequency of *KRAS* mutations in CRC is 36 – 40% (Amado et al., 2008). According to catalogue of somatic mutations in cancer (cosmic) (https://cancer.sanger.ac.uk/cosmic), frequency of G12C mutation among KRAS-mutated CRC is 7.9%. The most common mutation (p. V600E) for *BRAF* was observed only one case which the frequency of BRAF mutations in CRC is 47 – 96% (Rizzo et al., 2010). The coexistence of *KRAS* and *BRAF* mutations was not found in our patient. On the basis of our result and previous study, there is a statistically meaningful association between *KRAS* mutations and male gender, the higher age of patients (more than 50 years), and tumor size of *pT3*, tumor type of adenocarcinoma mucinous, and lymph nodes un-involvement (Walshe et al., 2017, Yang et al., 2017). The main limitations of our study were incomplete analysis of identified novel variant due to the expired patient. The patient that carry the novel variant had no siblings. We found that p. Ser616Thr (c.1846T>A) mutation was not previously reported. As well as other new variants, can be identified in *KRAS* and *BRAF* which lead to colorectal cancer. We conclude that other new mutations can be identified in the genes responsible to colorectal cancer such as *KRAS* and *BRAF* which may lead to this cancer.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

The authors gratefully appreciate the collaboration of school of medicine, Iran University of Medical Sciences. We acknowledge all employees and officials of Firoozgar Hospital, Iran University of Medical Sciences for helping us in this research project. Also, we thankfully appreciate

the patient family for their contribution to this study.

Funding

This work was supported by grants from Iran University of Medical Sciences project number 26544.

Competing interests

The authors declare there is no conflict of interest.

References

Adzhubei IA, Schmidt S, Peshkin L, et al (2010). A method and server for predicting damaging missense mutations. *Nat Methods*, **7**, 248-9.

Amado RG, Wolf M, Peeters M, et al (2008). Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol*, **26**, 1626-34.

Armaghany T, Wilson JD, Chu Q, Mills G (2012). Genetic alterations in colorectal cancer. *Gastrointest Cancer Res*, **5**, 19-27.

Asghari-Jafarabadi M, Hajizadeh E, Kazemnejad A, Fatemi SR (2009). Site-specific evaluation of prognostic factors on survival in Iranian colorectal cancer patients: a competing risks survival analysis. *Asian Pac J Cancer Prev*, **10**, 815-21.

Bray F, Ferlay J, Soerjomataram I, et al (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*, **68**, 394-424.

Choi Y, Chan AP (2015). PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics*, **31**, 2745-7.

Clarke CN, Kopetz ES (2015). BRAF mutant colorectal cancer as a distinct subset of colorectal cancer: clinical characteristics, clinical behavior, and response to targeted therapies. *J Gastrointest Oncol*, **6**, 660-7.

De Roock W, Biesmans B, De Schutter J, Tejpar S (2009). Clinical biomarkers in oncology: focus on colorectal cancer. *Mol Diagn Ther*, **13**, 103-14.

Dhomen N, Marais R (2007) New insight into BRAF mutations in cancer. *Curr Opin Genet Dev*, **17**, 31-9.

Dobre M, Dinu DE, Panaitescu E, et al (2015). KRAS gene mutations - prognostic factor in colorectal cancer?. *Rom J Morphol Embryol*, **56**, 671-8.

El-Shami K, Oeffinger KC, Erb NL, et al (2015). American Cancer Society Colorectal Cancer Survivorship Care Guidelines. *CA Cancer J Clin*, **65**, 428-55.

Faghani M, Fakhrieh Asl S, Mansour-Ghanaei F, et al (2012). The Correlation between Microsatellite Instability and the Features of Sporadic Colorectal Cancer in the North Part of Iran. *Gastroenterol Res Pract*, **2012**, 756263.

Fearon ER, Vogelstein B (1990). A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759-67.

Harada K, Hiraoka S, Kato J, et al (2007). Genetic and epigenetic alterations of Ras signalling pathway in colorectal neoplasia: analysis based on tumour clinicopathological features. *Br J Cancer*, **97**, 1425-31.

Jayasekara H, MacInnis RJ, Williamson EJ, et al (2017). Lifetime alcohol intake is associated with an increased risk of KRAS+ and BRAF-/KRAS- but not BRAF+ colorectal cancer. *Int J Cancer*, **140**, 1485-93.

Kircher M, Witten DM, Jain P, et al (2014). A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet*, **46**, 310-5.

Koochak A, Rakhshani N, Karbalaie Niya MH, et al (2016). Mutation Analysis of KRAS and BRAF Genes in Metastatic Colorectal Cancer: a First Large Scale Study from Iran. *Asian Pac J Cancer Prev*, **17**, 603-8.

Kranenburg O (2005). The KRAS oncogene: past, present, and future. *Biochim Biophys Acta*, **1756**, 81-2.

Kumar P, Henikoff S, Ng PC (2009). Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc*, **4**, 1073-81.

Larki P, Gharib E, Yaghoob Taleghani M, Khorshidi F, Nazemalhosseini-Mojarad E, Asadzadeh Aghdaei H (2017). Coexistence of KRAS and BRAF Mutations in Colorectal Cancer: A Case Report Supporting The Concept of Tumoral Heterogeneity. *Cell J*, **19**, 113-7.

Li FH, Shen L, Li ZH, et al. (2010). Impact of KRAS mutation and PTEN expression on cetuximab-treated colorectal cancer. *World J Gastroenterol*, **16**, 5881-8.

Maresso KC, Tsai KY, Brown PH, et al (2015). Molecular cancer prevention: Current status and future directions. *CA Cancer J Clin*, **65**, 345-83.

McCoy MS, Toole JJ, Cunningham JM, et al (1983). Characterization of a human colon/lung carcinoma oncogene. *Nature*, **302**, 79-81.

Nistal E, Fernandez-Fernandez N, Vivas S, Olcoz JL (2015). Factors Determining Colorectal Cancer: The Role of the Intestinal Microbiota. *Front Oncol*, **5**, 220.

Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. (2019). CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res*, **47**, D886-94.

Richards S, Aziz N, Bale S, et al (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*, **17**, 405-24.

Rizzo S, Bronte G, Fanale D, et al (2010). Prognostic vs predictive molecular biomarkers in colorectal cancer: is KRAS and BRAF wild type status required for anti-EGFR therapy?. *Cancer Treat Rev*, **36**, 56-61.

Schubbert S, Shannon K, Bollag G (2007). Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer*, **7**, 295-308.

Schwarz JM, Cooper DN, Schuelke M, Seelow D (2014). MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods*, **11**, 361-2.

Shihab HA, Gough J, Cooper DN, et al (2013). Predicting the functional, molecular, and phenotypic consequences of amino acid substitutions using hidden Markov models. *Hum Mutat*, **34**, 57-65.

Siegel RL, Miller KD, Fedewa SA, et al (2017). Colorectal cancer statistics, *CA Cancer J Clin*, **67**, 177-93.

Van Cutsem E, Kohne CH, Lang I, et al (2011). 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*, **29**, 2011-9.

Venselaar H, Te Beek TA, Kuipers RK, Hekkelman ML, Vriend G (2010). Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. *BMC Bioinformatics*, **11**, 548.

Walshe M, Moran R, Boyle M, et al (2017). High-risk family colorectal cancer screening service in Ireland: Critical review of clinical outcomes. *Cancer Epidemiol*, **50**, 30-8.

Waterhouse A, Bertoni M, Bienert S, et al (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res*, **46**, 296-303.

Wolpin BM, Meyerhardt JA, Mamon HJ, Mayer RJ (2007) Adjuvant treatment of colorectal cancer. *CA Cancer J Clin*, **57**, 168-85.

Yang M, Xiao X, Xing X, et al (2017). KRAS and VEGF

gene 3'-UTR single nucleotide polymorphisms predicted susceptibility in colorectal cancer. *PLoS One*, **12**, e0174140.
Zhao Q, Xu L, Sun X, et al (2017). MFG-E8 overexpression promotes colorectal cancer progression via AKT/MMPs signalling. *Tumour Biol*, **39**, 1010428317707881.