

# KRAS mutations in patients with colorectal cancer in Libya

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**Abstract.** Large prospective clinical trials have demonstrated that colorectal cancers (CRCs) with wild-type KRAS respond favorably to anti-epidermal growth factor receptor treatment, thus making mutational analysis obligatory prior to treatment. In our study, frozen CRC tissues from Libyan patients were analyzed for KRAS and HRAS mutations in codons 12/13 by direct sequencing and the correlations with clinical and pathological parameters were investigated. A total of 34 CRC cases, comprising 19 men and 15 women (age range, 24-87 years), were subjected to systematic analysis for RAS mutations. Although HRAS mutations were not detected in any of the patients in the study group, KRAS codon 12/13 mutations were present in 38.2% (13/34) of the patients. The frequent types of codon 12 mutations were glycine to aspartate (G12D, 46.1%); glycine to valine (G12V, 30.8%) and glycine to cysteine (G12C, 15.4%), while the codon 13 mutations were glycine to aspartate (G13D, 7.7%). G→A mutations occurred in 53.8% (7/13) of the patients, while G→T mutations occurred in 46.2% (6/13) of the patients. Mutations occurred at the first base of codon 12 or 13 in 2/13 (15.4%) and at the second base in 11/13 (84.6%) patients. There was no significant association between clinicopathological characteristics and KRAS mutation status, except the site of the tumors harboring KRAS mutations, which was as follows: The frequency was higher among tumors located in the left colon (8/13, 61.5%) compared to other sites ( $P=0.027$ ). KRAS mutations were correlated with advanced age, with 10/13 being aged >50 years and affected 8/15 female patients (53%) compared with 5/19 male patients (26%). The highest frequency of KRAS mutations was observed in highly differentiated CRCs (8/13).

## Introduction

Colorectal cancer (CRC) is the 3rd and 4th most commonly diagnosed cancer globally in women and men, respectively (1-3). The transformation of the normal colonic epithelium is the result of the progressive accumulation of genetic and epigenetic alterations that promote tumor growth. Colon cancer cells result from a multi-step progression in molecular and morphological changes of normal cells. In addition, genetic instability and germ line genetic defects drive the initiation of sporadic colon cancers (4-6).

The RAS oncogene family, comprising three known human isoforms, NRAS, HRAS and KRAS, regulates a number of cellular functions, including cell proliferation, apoptosis, migration and differentiation (7). KRAS mutations comprise 86% of all RAS mutations in human cancers (8). The frequency of KRAS mutations is the highest (21.6%) in all cancers, followed by NRAS (8.0%) and HRAS (3.3%) (9). The hotspot of KRAS mutations is located in exon 2, and the most frequent changes are identified in codons 12 (~82% of all reported KRAS mutations) and 13 (~17%). Other mutations of KRAS have been detected in codons 61 and 146, but these have little or no effect on the progression of CRC (10). The most frequent change is the transition of GGT to GAT in codon 12, specifically DNA nucleotide mutations involving G→A or G→T (7). However, 60% of NRAS mutations occur mainly in codon 61 vs. 35% in codon 12, while HRAS mutations in CRC show an intermediate pattern with an approximate 50:40 split between mutations in codons 12 and 61, respectively (11). KRAS and HRAS mutations comprise single-nucleotide point mutations, with the most common substitutions in KRAS resulting in G12D, G12A, G12R, G12C, G12S, G12V and G13D substitutions and the most frequent codon 12 substitutions being G12D and G12V (10). However, in HRAS, the most common mutation results in a G12V substitution (11). The presence of a glycine residue in codon 12, therefore, plays an important role in the normal function of the RAS proteins. This single-nucleotide substitution that results in the replacement of glycine with another amino acid leads to the formation of a constitutively active GTPase (12).

RAS proteins are activated when nearby transmembrane receptors, such as growth factor receptors, G-protein coupled receptors and toll-like receptors, are bound by the corresponding ligand. The subsequent intracellular signaling cascade involves guanine exchange factors (GEF) that facilitate the activation of RAS by replacing the inactive GDP with GTP. RAS activation

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triggers the downstream activation of a wide variety of effectors, including serine/threonine kinases, GTPase-activating proteins (GAPs), phosphoinositide 3-kinase and GEFs. RAS is deactivated when the GTP is hydrolyzed to GDP. When GTP binds to the KRAS protein, KRAS undergoes a conformational change involving two regions of the protein known as Switch 1 (amino acids 30-38) and Switch 2 (amino acids 59-67), which form an effector loop controlling the specificity of the binding of this GTPase to its effector molecules. The changes in KRAS conformation affect its interactions with multiple downstream transducers, such as GAPs, which amplify the GTPase activity of RAS 100,000-fold (13). Once KRAS is mutated, it becomes constitutively active and the regulation of downstream functions is lost, resulting in unregulated cell growth (7). Screening of KRAS mutations is widely used in clinical practice to decide on the treatment for CRC (14). Recently, KRAS mutation screening has become common practice before prescribing anti-epidermal growth factor receptor monoclonal antibodies in the treatment of advanced CRC (15).

CRC is the second most common type of cancer among patients admitted to the National Cancer Institute (NCI)-Misurata, where ~180 new cases (52% male and 48% female patients) were admitted in 2019. The incidence and mortality rates for CRC increase with age, with >90% of the patients aged >50 years (2). The aim of the present study was to investigate the presence of KRAS and HRAS mutations among patients with CRC in NCI-Misurata, in order to determine whether the frequency of those mutations is associated with the type and stage of CRC.

## Materials and methods

**Tissue samples.** The present study included 34 frozen tumor tissue samples obtained from patients with CRC, who underwent curative surgery at NCI-Misurata. The patients were diagnosed at the Department of Histopathology between December 2016 and August 2017. The study protocol was approved by the Ethics Committee of NCI-Misurata. All tumor tissues were placed on ice immediately upon removal from the patient, sectioned and stored at -80°C until DNA was extracted.

**Clinical records.** The clinical and demographic data collected from patient records included: Age, sex, family history of CRC, tumor site, degree of tumor differentiation, tumor stage and histological type, such as common adenocarcinoma or mucinous carcinoma. Patients with family history of CRC were selected for the present study. All the patients have signed a consent form prior to the use of the studied tissues, according to the regulations of the NCI-Misurata.

**DNA extraction and PCR reaction.** Genomic DNA extraction from frozen tumor tissues was carried out using QIAamp DSP DNA Fresh Tissue kit (Qiagen GmbH). After the washing steps, the DNA was eluted in Tris/EDTA buffer and stored at -20°C. The DNA was quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). After extraction of the genomic DNA from the samples, the KRAS and HRAS exons 1 and 2 were amplified using a PCR SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific, Inc.) using the forward 3'-AAGGCCTGCTGAAAATGACTG-5' and reverse 3'-CAAAGAATGGTCCTGCACCAG-5' primers for KRAS-exon 2

and the forward 3'-GGGCCCTCCTTGGCAGGTGG-5' and reverse 3'-CACCTGGACGGCGGCCAG-5' primers for HRAS-exon 2. Each PCR reaction was performed in a volume of 20 µl containing PCR buffer, 2.5 mM MgCl<sub>2</sub>, 5 units of Taq polymerase, 1 mM of each dNTP, forward and reverse primers (10 pmol) and 100 ng of genomic DNA. The PCR reaction ran with the following program: 95°C initial denaturation for activating the Fast Star Taq DNA Polymerase for 5 min; step 2: Denaturation at 95°C for 30 sec; step 3: Annealing of the primers to the template at 54°C for HRAS in exons 1 and 2, 45°C for KRAS in exon 1, and 43°C for exon 2 for 30 sec; and step 4: 3 min at 72°C for primer extension. Steps 2-4 were repeated 25 times, followed by a final extension step at 72°C for 5 min; subsequently, the PCR reaction was cooled down to 8°C. The PCR products were subjected to direct DNA sequencing.

The amplification product sizes were confirmed by electrophoresis on 1% agarose gel in Tris-borate-EDTA buffer (Sigma-Aldrich; Merck KGaA), and visualized on a UVP BioSpectrum™ 500 Imaging System (VWR Corporation). The PCR reaction products were purified using the MinElute PCR Purification Kit (Qiagen GmbH) and subjected to sequence analysis.

**Direct DNA sequencing.** Direct sequencing of PCR products was performed after purification with the PCR Product Purification Kit (Roche Diagnostics GmbH). The forward primers used in PCR amplification of KRAS and HRAS genes were used for sequencing in on the EVO150 Genetic Analyzer (Tecan Group, Ltd./Applied Biosystems; Thermo Fisher Scientific, Inc.). To ensure the accuracy of sequencing results, all the molecular tests and the direct sequencing analysis were performed twice on each sample. All DNA sequencing reactions were performed by Inqaba Biotec.

**Statistical analysis.** Data analysis was performed using SPSS version 17.0 (SPSS Inc.). The Fisher's exact test was used for comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**KRAS and HRAS mutations in histopathological biopsies.** KRAS mutations were identified in 13/34 (38.2%) of the tumors, while all the patients in this study group had the wild-type HRAS gene (Fig. 1). The site of the tumor was found to be associated with KRAS mutations ( $P = 0.027$ ). Tumors located in the left colon had a significantly higher frequency of mutant KRAS status (8/11; 72.7%) compared with those located in the right colon. Table I shows the frequency and association with clinicopathological characteristics of patients who harbored mutant and wild-type KRAS tumors; 76.9% (10/13) of the patients with tumors harboring mutant KRAS presented with stage B, C or D colon cancer, indicating that patients with mutant KRAS tumors are more likely to have advanced-stage disease. Tumor differentiation status, sex, patient age and stage at diagnosis were not identified as significant predictors of KRAS status ( $P = 0.458$ ).

**Type and frequency of KRAS gene mutations.** The type and frequency of KRAS gene mutations are detailed in Table II.

Table I. Distribution of tumour characteristics according to KRAS status.

Characteristics	KRAS mutation status			P-value
	Overall	Mutant	Wild-type	
Number of patients	34	13	21	
Sex				0.107
Male	19	5	14	
Female	15	8	7	
Age (years)				0.524
≤50	10	3	7	
>50	24	10	14	
Localization				0.027
Right colon	11	3	8	
Left colon	11	8	3	
Rectosigmoid	4	0	4	
Rectum	8	2	6	
Differentiation				0.458
High	16	8	8	
Moderate	12	4	8	
Low	2	0	2	
Unknown	4	1	3	
Duke's stage				0.561
A	3	2	1	
B	7	4	3	
C	18	5	13	
D	3	1	2	
Unknown	3	1	2	

Table II. Distribution of KRAS mutation types.

Codon	Amino acid substitution	Incidence, %	Number of samples	Base change
12	Gly12Asp	46.1	6	GGT>GAT
12	Gly12Val	30.8	4	GGT>GTT
12	Gly12Cys	15.4	2	GGT>TGT
13	Gly13Asp	7.7	1	GGC>GAC

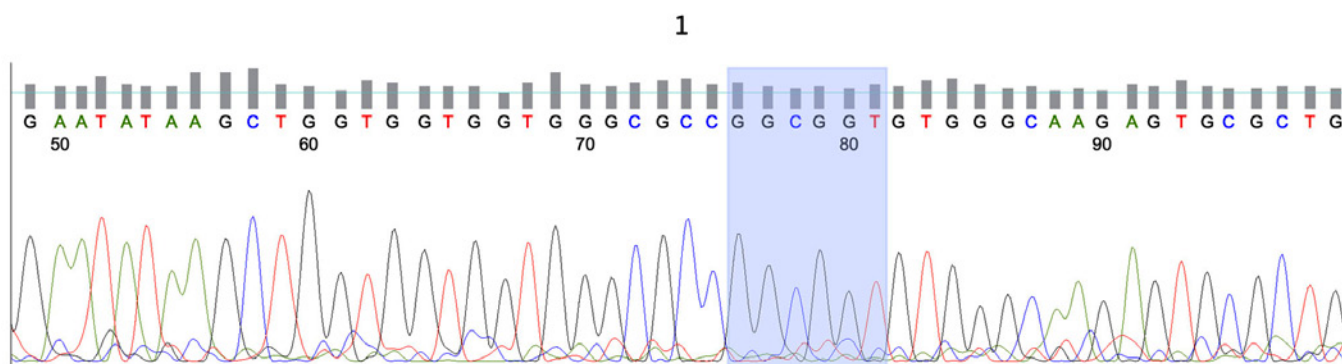


Figure 1. Sanger sequencing electropherogram showing the wild-type HRAS codons 12 and 13 (GGCGGT). No HRAS mutations were detected in any of the patients in the cohort.

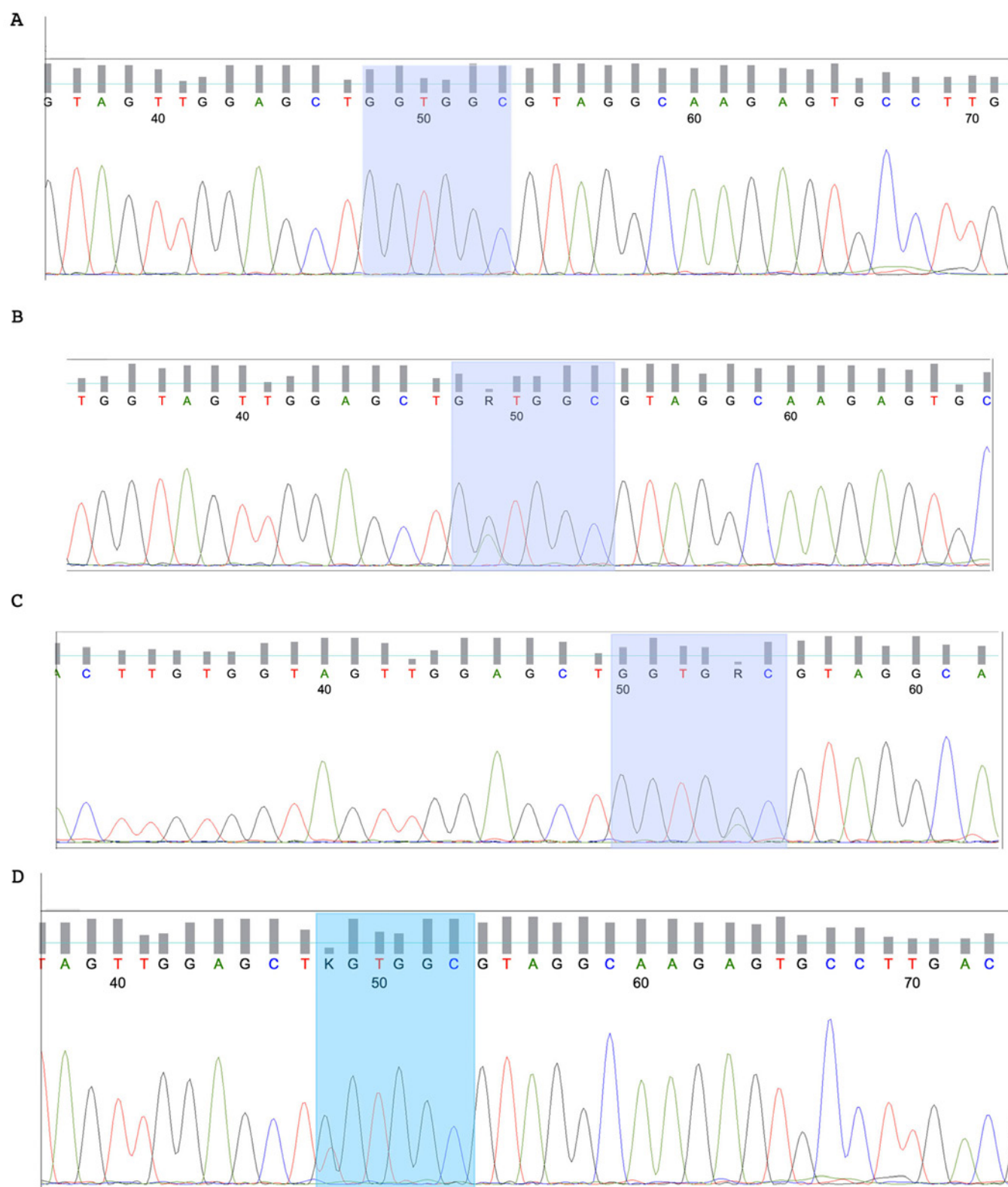


Figure 2. DNA isolated from patients was amplified by PCR using primers specific for codons 12/13 of the KRAS gene. The PCR products were then subjected to Sanger sequencing and the results are presented as follows: (A) wild-type KRAS, (B) codon 12 GGT>GAT (GLY>ASP), (C) codon 13 GGC>GAC (GLY>ASP) and (D) codon 12 GGT>TGT (GLY>CYS).

Of the 13 tumors, 12 (92.3%) had mutations in codon 12, while 1 had a mutation in codon 13 (7.7%). Of the 13 patients with CRC harboring KRAS mutations 6 (46.1%) had a Gly12Asp mutation 4 (30.8%) had a Gly12Val mutation, 2 (15.4%) had a Gly12Cys mutation, and 1 (7.7%) had a Gly13Asp

mutation. Mutations from G to A occurred in 7/13 (53.8%) of the patients, and from G to T in 6/13 (46.2%) of the patients (Fig. 2). Mutations occurred at the first base of codon 12 or 13 in 2/13 (15.4%) and at the second base in 11/13 (84.6%) of the patients (Table II).

## Discussion

The present study investigated the association between clinicopathological factors and KRAS mutation status in Libyan patients with CRC and, except for the tumor site, no other significant associations were identified. This observation is consistent with the findings of a Saudi Arabian study (16) and the RASCAL multicenter study (17), which demonstrated a trend for higher mutation frequency in tumors of the left colon (51 and 58.8%, respectively) compared to other sites (16,17). This finding has been attributed to the increased exposure of the left-sided bowel lumen to ingested carcinogens and mutagens. By contrast, KRAS mutations have been reported to be slightly more frequent in the right colon compared with other sites (47 vs. 41%, respectively) in studies from Saudi Arabia (18,19), while another study from Turkey reported that the KRAS mutation frequency was significantly higher in tumors located in the ascending colon (20).

There are several factors that may explain the variations in the observed frequencies of KRAS mutations according to tumor location in different studies. These include the use of different methodologies for the detection of mutations, with different ranges of sensitivity and specificity. Environmental factors have a major impact on different populations with diverse lifestyles, dietary habits, and variable exposures to carcinogens. Furthermore, the genetic polymorphisms in the carcinogen-metabolizing genes in different population groups must also be taken into consideration (21-24).

KRAS mutations were identified in 38.2% of all CRC patients in our study group. This finding was in agreement with previous reports that identified KRAS mutations in 30-40% of patients with CRC (17,21). Consistent with previous reports, 92.3% of the studied tumors had a single base mutation at codon 12 of the KRAS gene, and 7.7% had a single base mutation at codon 13 (17,21,22). The most common single base mutation (46.1%) was a GGT to a GAT transition in codon 12 that resulted in a change from the amino acid glycine to aspartic acid (Gly12Asp). The frequency of KRAS mutations in our study group did not differ when compared with those of most other studies in Europe and the United States (23,24). Previous studies that enrolled a relatively large number of Arabian patients with different stages of the disease, reported KRAS mutation rates of 28-56% in Saudi Arabia (19,25-27), 23-32% in Tunisia (22,28,29), 33-44% in Jordan (30,31) and 42% in Egypt (32).

A number of factors may be associated with variations in the observed frequencies of KRAS mutations in different studies. Among these, environmental factors may be the most important parameter that may explain the difference in KRAS mutations between Libya and neighboring countries, such as Tunisia and Egypt, and western countries.

In conclusion, KRAS genetic alterations in codons 12 and 13 were found to be higher in this group of Libyan patients with CRC. However, further studies are required to fully elucidate the molecular background of CRC, by investigating other genes, such as APC, BRAF and NRAS, as well as other exons in the KRAS, HRAS and NRAS genes. Future studies of larger patient groups may provide more accurate information regarding the association between KRAS, HRAS and NRAS mutations and the clinicopathological characteristics of patients with CRC.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

AA conducted the experiments and the data analysis. MD assisted with the analysis of the obtained results. MA provided the clinical data of the patients with CRC. MIP was the co-supervisor of the study and critically revised the manuscript for important intellectual content. OA supervised the project, experimental design and data analysis, and edited the manuscript. MIP and OA have assessed and confirmed the authenticity of raw data. All the authors have read and approved the final version of the manuscript.

## Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the National Cancer Institute-Misurata, the institutional research committee, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients have signed the consent prior to the use of the studied tissues according to the regulations of the NCI-Misurata.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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