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Prevalence and Pattern of KRAS and NRAS Mutations in Colorectal Cancer: A Libyan Retrospective Study

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

About 53% of colorectal cancer (CRC) patients were reported to have the Kirsten rat sarcoma viral oncogene homolog (KRAS) gene mutations. These mutations in the KRAS gene are able to render the targeted therapy agents such as monoclonal antibodies cetuximab and panitumumab ineffective against the epidermal growth factor receptor (EGFR). Disparities between regions have been described in the literature regarding these mutations. This is an original investigation aimed at characterising the frequency and patterns of KRAS mutations in Libyan patients with colorectal cancer. Tissue samples of 79 cases of colorectal cancer were analysed for KRAS and NRAS mutations. Of these, 44 (55.7%) reported positive. In the KRAS positive patients, there were 23 (52.3%) males and 21 (47.7%) females. Majority of cases (77.0%) were with point mutations in codon 12 whereas (7%) had a single mutation in codon 13. There were 3 patients showing mutation

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in codon 61 with two nucleotide changes whereas the last 4 patients exhibited three nucleotide changes in codon 146. The more prevalent KRAS mutation was p.Gly12Val (c.35G>T) (29.5%), followed by p.Gly12Asp (c.35G>A) (25%). The G>A transitions in both codons 12 and 13 accounted for 41.0% of all the mutant KRAS cases. The transversions G>T in codon 12 alone forms 38.6% of the total KRAS mutation. The p.Gly12Val (c.35G>T) mutation had the highest frequency in both males (26.1%) and females (33.3%). Same tendency also was seen in p.Gly12Asp (c.35G>A) mutation but to lesser extent with (30.4%) in male and (19%) in female. Interestingly, the p.Gly13Asp (c.38G>A) mutation had pure appearance in male. Multiple mutations in the same individual were detected in 7 patients in this cohort (16%). Our results showed a relatively higher prevalence of KRAS mutation in Libyan patients compared to other analogous data observed worldwide. Two samples only out of 29 showed mutation in NRAS codon 61. Being a retrospective study with small sample size were the main limitations for this study. Thus we recommend that conduction of larger studies is needed in the future.

Keywords: KRAS; NRAS; mutation; colorectal cancer (CRC); prevalence; Libyan patients.

1. INTRODUCTION

Libya was found to have the highest colorectal cancer incidence rate in North Africa. This cancer was the second among the most common malignancies in Libya being 19% of them, with variation in its incidence in the various cities [1]. While the precise causes of this alarmingly high rate are unknown, genetic susceptibility, greater Westernization of the Libyan diet, physical inactivity, and an absence of screening programs could all be contributing factors. In some areas, Libyan diet is traditional, while in others, it is modernized (Westernized). This reflects the existing disparities between rural and urban areas. It is expected that the incidence of CRC will rise as consumption of Western-style cooking increases and traditional food consumption decreases. However, determining such a trend will necessitate a long-term study. A high prevalence of diabetes mellitus, smoking, obesity, and other risk factors exist in Libyan society. Late presentation is a major issue in the Libyan scenario. This could be due to a variety of factors, including absence awareness and social stigma. Rural areas face transportation challenges [1].

The function of the two monoclonal antibodies cetuximab and panitumumab has been established as targeted therapies working against the epidermal growth factor receptor (EGFR), when used as adjuvant therapy for a cancer in its advanced stage along with cytotoxic chemotherapy [2-4]. EGFR is reported to show an overexpression in around 50–80% of colorectal cancers. Accordingly, these agents are developed to interfere with activation of ligand-induced EGFR tyrosine kinase and, therefore, block downstream signalling [5]. In clinical practice and in spite of improvements in molecular targeted therapies to colorectal cancer, EGFR, the therapeutic target of such kind of drugs, did not achieve the expected predictive curative effect. Only the colorectal cancer with wild-type Kirsten rat sarcoma viral oncogene homolog (KRAS) reacts to anti-EGFR antibodies therapy, whereas there is no therapeutic reaction seen in colorectal cancer with KRAS mutations [5-7]. The KRAS gene is a significant component of the EGFR signalling pathway. Previous studies have shown that in about 35–45 per cent of colorectal cancer patients, mutations can appear within KRAS gene in exon 2 codons 12 and 13, making EGFR inhibitors unable to carry out their action [8,9]. The use of EGFR-targeted monoclonal antibody therapy with cetuximab and panitumumab is acknowledged by the US Food and Drug Administration (FDA) in patients with colorectal cancer and this should be accompanied by determination of status of KRAS mutation that considered to be a biological predictor for resistance [10-12]. Also, the American Society of Clinical Oncology and the European Society for Medical Oncology have approved important recommendations that the use of these antibodies be limited to patients with KRAS wild-type colorectal cancers [13,14]. The KRAS gene is in the Ras family of oncogenes, which also comprises two other genes: HRAS and NRAS. These three genes produce proteins called GTPases that play significant roles in division, differentiation, and the self-destruction of cells (apoptosis).Thus, KRAS gene mutation detection has significant clinical relation for the purpose of designing individualized therapeutic strategies for patient [15]. Also, NRAS gene can host other mutations that have been linked to clinical resistance to anti-EGFR monoclonal antibodies (mAbs). Frequent locations of KRAS mutations are found in codons 12, 13 (exon 2), 59, 61

(exon 3), 117 and 146 (exon 4). NRAS mutations can appear on exons 2, 3 and 4 (including amino-acids 12–13, 59–61 and 117–146, respectively).

RAS mutations are accounting for almost 53% of colorectal cancer cases, in which KRAS exon 2 mutations represent 42% and the rest which is 11% represented by KRAS outside exon 2 and NRAS mutations [4]. On the other hand, some mutations appear at lower frequency, less than 5% of all mutation types, in colorectal cancer in codons 2, 3, 4, 63 and 154 [4, 8].

Researches of colorectal cancers have shown that the majority of mutations reported are single nucleotide point mutations, where the most frequent are G > A transitions and G > T transversions [16]. p.Gly12Asp, and p.Gly12Val are the most common substitutions in the codon 12 mutations, whereas codon 13 mutations show increased frequency in substitution of glycine for aspartate (p.Gly13Asp) [4]. Regional differences among these mutations have been described in the literature, so it is important to explore and recognize the underlying main patterns of them in the KRAS gene in different population groups of metastatic colorectal cancer. For instance, other mutations of KRAS that are infrequent has also been detected in Chinese cases in codons 45, 69 and 80 [17,18].

Data about KRAS and NRAS genes and their different mutations could be useful in colorectal cancer case selection for the anti-epidermal growth factor receptor targeted therapies as well as for making available affordable targeted tests for prevailing mutations at the national level. This is an original investigation which aims to characterise the frequency and patterns of KRAS and NRAS mutations in Libyan patients with colorectal cancer referred to the Pathology Department at National Cancer Institute (NCI) of Misrata city in Libya for KRAS mutation analysis or subsequently to NRAS testing in case where KRAS analysis was negative. The small sample size of the cohort was the main limitation for this study. Therefore, conduction of larger studies about KRAS and NRAS genotype is recommended in Libyan patients with colorectal cancer.

2. METHODS

The sex, age, site and KRAS genotypes identified in colorectal cancer patients referred from all regions of Libya for KRAS mutation analysis to the pathology department at national cancer institute (NCI) of Misrata city in Libya from January 2018 to April 2020 were registered in Excel sheet database. This database was retrospectively analysed.

Genomic DNA was obtained from formalin-fixed paraffin-embedded (FFPE) tissue sections of colorectal tumours that are previously confirmed by the histopathology examination using Idylla™ KRAS Mutation system according to the manufacturer's protocol. The tumour samples were specially selected by a pathologist to assure that it is empty from any substantial defects, necrosis or inflammation and contains enough amount of tumour cells to be analysed.

The Idylla™ KRAS Mutation Assay allows detection of mutation in three exons named 2, 3, and 4 of the KRAS oncogene. This test performs a group of five allele-specific multiplex PCR reactions, developed for the specific amplification of KRAS gene sequences that are including a mutation in codons 12, 13, 59, 61, 117, or 146 [19]. Thus, this assay can detect twenty one mutations in KRAS oncogene as the following: seven mutations in exon 2 (codons 12 and 13), nine mutations in exon 3 (codons 59 and 61), and five mutations in exon 4 (codons 117 and 146). In the event of several mutations are present, just the predominantly identified change, lowest ΔCq value, is given [19].

The Idylla™ instrument (Biocartis, Mechelen, Belgium) is fully automated machine and its use to carry out the assay does not necessitate a pre-treatment of the sample such as manual dewaxing of paraffin or formalin-fixed paraffin-embedded (FFPE) pre-processing. The process of KRAS mutation detection is passing through a group of steps: the FFPE samples were inserted into the cartridge of Idylla™ platform, after that the cartridge is placed into the device where the sample is exposed to a series of reagents, enzymes, heat, and high intensity focused ultrasound (HIFU) resulting in dewaxing of the sample and finally rupture of the tissue and break down of the cells that leads to release of the nucleic acids for following real-time PCR amplification [19]. In each of the five multiplex PCR reactions, a sample processing control, including the concurrent amplification of a monitored section in the junctional region of intron 4/exon 5 of the KRAS gene, was conducted to verify for appropriate carrying out of the entire sample to-result process and as a gauge to assess the availability of the amplifiable

amount of DNA in the used tissue sample [19]. Using the same methodology, a subset of the used samples, consisting of 29 cases out of 35 found to be wildtype for KRAS codons 12 and 13, 61 and 146 were subsequently subjected to mutational analysis for NRAS codons 12, 13, and 61. No data was available for the remaining 6 cases.

A quantification cycle (Cq) value is calculated by the IdyllaTM software in each successful PCR curve. The KRAS mutation status is regarded as positive if the difference between the calculated Cq for a KRAS mutant PCR signal and the KRAS wildtype Cq value, the ΔCq value, is within a range of approved values and consequently the specific mutation or mutation group is reported. On the other hand, in case the used tissue sample showing a valid KRAS wild-type signal but a ΔCq value outside the validated range is characterised as being negative (wild-type) for KRAS mutation [19].

Invalid results appear as a consequence of several causes such as the improper sample insertion inside the cartridge, inappropriate size of the tissue sample, presence of inhibitors in the sample or inadequate amplifiable DNA. Other factors related to the cartridges themselves can cause invalid results including incorrect storage of cartridges, use of cartridges that surpassed their allowed period to use after removal from their coverages, or defected cartridges [19].

2.1 Statistical Analysis

All data were tabulated, and statistical analyses were performed in regards to the KRAS genotype and clinicopathological variables namely age groups and gender using the Statistical Package for Social Sciences (SPSS) for Windows version 22.0 (SPSS statistics 22). The frequencies and statistics for the different parameters were studied by using a descriptive analysis. Associations between variables were tested through Fisher's Exact Test and Chi-square test from which tables and graphs were generated. All p values below 0.05 were considered statistically significant.

3. RESULTS

Tissue samples of 79 cases of colorectal cancer were analysed for KRAS mutations. Of these, 44 (55.7%) reported positive. The remaining 35 (44.3%) samples reported absence of mutation. Overall, there were 39 (49.4%) males and 40 (50.6%) females. Among the KRAS positive cases, there were 23 (52.3%) males and 21 (47.7%) females. Their ages ranged from 32 to 70 years with a median age of 51.14 years. There were 34 patients (77.0%) with point mutations in codon 12 while 3 (7%) had a single mutation in codon 13. There were 3 patients showing mutation in codon 61 with two nucleotide changes whereas the last 4 patients exhibited three nucleotide changes in codon 146. The most common KRAS mutation was p.Gly12Val (c.35G>T) (29.5%), followed by p.Gly12Asp (c.35G>A) (25%). The distribution of the different KRAS mutations identified in Libyan CRC patients is shown in Table 1.

The G>A transitions in both codons 12 and 13 accounted for 41.0% of all the mutant KRAS cases. However, the transversions G>T in codon 12 alone forms 38.6 of the total KRAS mutation. Fig. 1 shows the percentage distribution of the mutant KRAS (G>A) transitions and (G>T) and (G>C) transversions identified in the Libyan cohort.

Table 1. Distribution of the different KRAS mutations identified in Libyan CRC patients

KRAS mutation type	Number of patients	Percentage
Codon 12		
p.Gly12Ala (c.35G>C)	2	4.5%
p.Gly12Cys (c.34G>T)	4	9%
p.Gly12Val (c.35G>T)	13	29%
p.Gly12Ser (c.34G>A)	4	9%
p.Gly12Asp (c.35G>A)	11	25%
Codon 13		
p.Gly13Asp (c.38G>A)	3	6.8%
Codon 61		
p.GIn61His (c.183A>C ; c.183A>T)	3	6.8%
Codon 146		
p.Ala146Pro / p.Ala146Thr / p.Ala146Val (c.436G>C / c.436G>A / c.437C>T)	4	9%
Total	44	100%

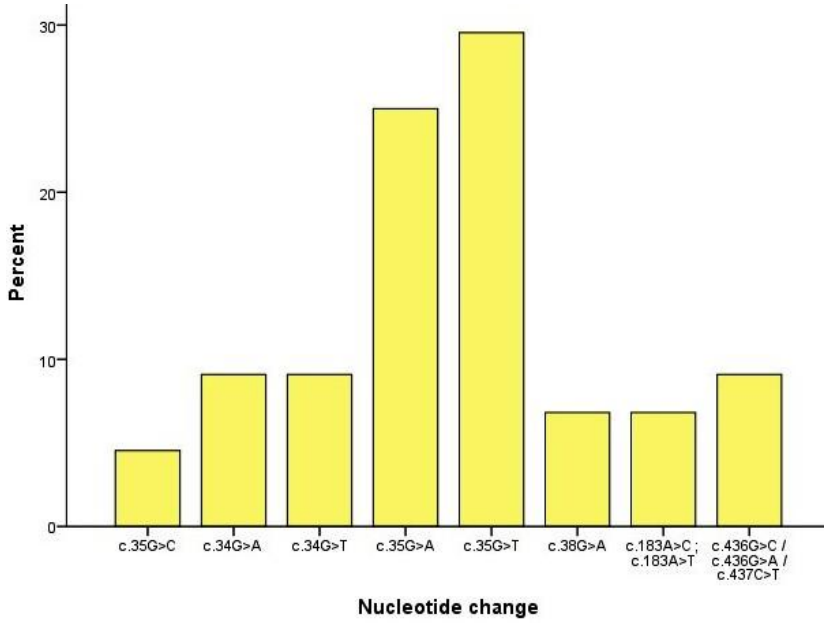

Fig. 1. Percentage distribution of the mutant KRAS (G>A) transitions and (G>T) and (G>C) transversions in the study cohort

No correlation was found between the KRAS mutation pattern and gender. The p.Gly12Val (c.35G>T) mutation had the highest frequency in both males (26.1%) and females (33.3%). Same tendency also have been seen in p.Gly12Asp (c.35G>A) mutation but to lesser extension with (30.4%) in male and (19%) in female. Interestingly, the p.Gly13Asp (c.38G>A) mutation had pure appearance in male, there was no contribution by female sex. The gender distribution of the KRAS genotypes is shown in Fig. 2.

No statistically significant difference was found between the KRAS genotypes and the gender (p = 0.698, Fisher's exact test).

The highest percentage of mutant KRAS cases was found in the 41–50 years age group, it is sole age group in which the genotype KRAS mutation p.Gly12Ala is detected. The stratification of the mutant KRAS genotypes according to age groups is shown in Fig. 3.

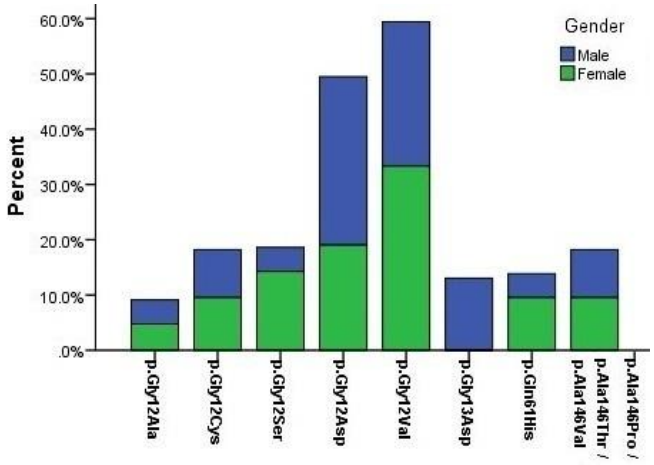

Fig. 2. Percentage distribution of the KRAS mutations according to gender

Interestingly, multiple mutations in the same individual were detected in 7 patients in this cohort (16%). Three of these 7 mutations were detected in codon 61 with p.GIn61His (c.183A>C; c.183A>T) and they were observed in only one age group 51-60 years whereas the other four appeared in codon 146 with p.Ala146Pro/p.Ala146Thr / p.Ala146Val (c.436G>C / c.436G>A / c.437C>T) and appeared in ages between 31-60 years. Mutational analysis for NRAS gene was carried out for the 29 cases found to be wildtype for KRAS analysis revealed presence of two samples only with mutation detected in NRAS codon 61. These two male and female samples showed a single nucleotide change with different mutation type in codon 61, Q61R and codon 61, Q61K respectively. There is five years difference between the ages of these two cases where the female case aged 70 year and the male case aged 76 year. Description of the mutations in these two samples is shown in Table 2.

Absence of correlation against clinico-pathological parameters of the cancer with a small sample size were the main limitations for this study. Furthermore, assessment of other vital biomarkers linked to mutations in KRAS exon 3 or 4, NRAS, or BRAF were not conducted. Hence we recommend that conduction of larger investigations is required about KRAS, NRAS, or BRAF genotype in Libyan patients with colorectal cancer and correlating them with other variables such as the clinicopathological parameters.

4. DISCUSSION

The overall frequency reported in this study was (55.7%) and this is not in accordance with what was observed for the KRAS mutation incidences in colorectal cancer patients worldwide. For example, Asia with 24%, Europe with 36% and South America with 40% [3]. This variance of KRAS mutation occurrence may appear as a result of different elements, such as thequality and quantity of FFPE tissue samples, availability of tumour cells in the used tissue sample, quality of the extracted DNA, the variability between KRAS assays used in the different laboratories and goal of the investigation (testing target). Also, this may reflect the genetic heterogeneity in

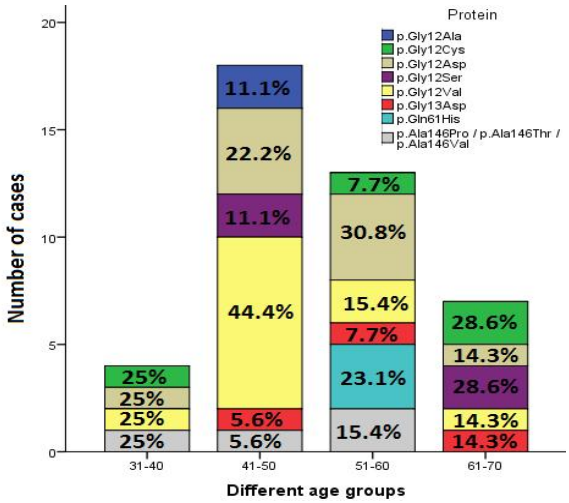

Fig. 3. Percentage distribution of the mutant KRAS mutations according to age groups

No statistically significant difference was found between the KRAS genotypes and the age groups (p = 0.155, Fisher's exact test)

Table 2. Details of NRAS mutations identified in two out of thirty five samples of Libyan CRC patients found to be wildtype for KRAS mutational analysis

Gender	Age	NRAS mutation type	Number of patients	Percentage
		Codon 61		
Male	76 year	p.GIn61Arg(c.182A>G)	1/29	3.457%
Female	70 year	p.GIn61Lys (c.181C>A)	1/29	3.457%

the KRAS mutation patterns in colorectal cancer patients. In addition to the previous factors, various pathogenetic ways at molecular level and environmental effects are other important factors to be considered [20]. However, although it is not obvious why this relatively higher prevalence of KRAS mutation is detected in Libyan patients, ethnicity and geographic location of the nation as North African country could contribute to that. One more factor which can affect the results is the testing technique and the type of specimen. For instance, as compared to the direct sequencing technique, it has been described that the Pyrosequencing technology, a real-time, does not require electrophoresis, nucleotide extension sequencing and next generation sequencing (NGS) techniques, are effective in wide range of applications. A study conducted on a cohort of 168 tumours using the pyrosequencing technique identified KRAS mutations in 30 tumours (17.9%) with KRAS wild-type using direct sequencing alone [21]. Accordingly, there is a perception that the low-frequency KRAS mutations detected by the direct sequencing technique can be explained by the presence of subclones that conceal KRAS mutations within genetically heterogeneous tumours [22]. Actually, it should be pointed out that about 20.0% of cases who showed wildtype KRAS status based on KRAS exon 2 test may conceal undetected extended RAS mutations in codons 59, 61 (exon 3), or codons 117 and 146 (exon 4) [23,24].

Earlier investigations [2, 3, 10, 11] have showed that the more prevalent KRAS mutation types are p.Gly12Asp, p.Gly12Val and p.Gly13Asp, accounting for just about 70.0% of all mutations. In our study, a different pattern was seen where p.Gly12Val (c.35G>T) (29%) and p.Gly12Asp (c.35G>A) (25%) were the most common mutations meanwhile p.Gly13Asp mutation forming only 6.8% of the overall mutations. The more frequent single mutation (29%) detected in our study was a G>T transition in codon 12 p.Gly12Val (c.35G>T) followed by G>A transition (25%) in the same codon p.Gly12Asp (c.35G>A). In comparison with a previous investigation conducted on cohort of 108 colorectal tissue samples in which the most common single mutation (40%) identified was a G>A transition in codon 13 [2]. Moreover, data from our cohort showed a comparatively lower frequency (4.5%) of the G>C transition in codon 12 (c.35G>C; p.Gly12Ala). In a Chinese study of colorectal cancer tumour samples using the DNA sequencing method the frequency of KRAS mutations was 33.3% (30/90) [4]. A comparable pattern was stated in Japanese cohort of 99 colorectal cancer cases where the frequency of KRAS mutations was registered to be 37.4% (37/99) [3]. They found that the more frequent mutation was the (p.Gly13Asp) mutation within codon 13 that detected in 11 (29.7%) of these cases. Conversely in codon 12, a higher frequency of the (p.Gly12Asp) mutation has been detected in 10 (27.0%) cases, whereas the GGT→GTT (p.Gly12Val) detected in 8 (21.6%) cases. Their findings are in parallel to present study but with some difference in the frequency particularly in codon 13.

KRAS somatic mutations were found in the colorectal cancer tissue samples of 31.5% (16/51) Tunisian patients where 81.2% showed presence of single mutation in codon 12 whereas 23.0% showed a single mutation in codon 13 [25]. According to their findings, 81.3% of mutations detected were transitions and (23%) were transversions and the more prevalent single mutation (50%) was a G>A transition in codon 12 (c.35G>A; p.Gly12Asp). This is not in agreement with our findings where transitions accounted for 34% in codon 12 whereas the transversions accounted for 42.5% in the same codon. Several studies conducted among Caucasian and Asian showed a superiority of the G>A transition in codon 12 (c.35G>A; p.Gly12Asp) [20, 26-31]. As opposed to these studies, the more frequent mutation detected in codon 12 in this study was p.Gly12Val (c.35G>T) (29%) followed by p.Gly12Asp (c.35G>A) (25%). An analogous outcomes was reported in a previous investigation carried out of 299 Indian patients suffering from colorectal cancer [11]. Similar to the findings observed in the Indian study [11], there was no strong association between the KRAS genotypes and the age and gender of the patients in our investigation. Number of researches described that the KRAS genotypes correlated strongly with age and gender whereas other researches did not note any important impacts between them [11, 18]. Nevertheless, because of the limitations of this study, firm outcomes about those variations could not be drawn.

5. CONCLUSION

The pilot results of our study show a prevalence of mutation in the KRAS gene in colorectal cancer cases at rate of 55.7%. On one hand,

such a large figure signifying the importance and need of conducting this test for targeted therapy management in Libyan colorectal cancer patients. On the other hand, testing KRAS gene status would help to control the use of EGFR inhibitor therapy through the right case selection that results in only the suitable patients receive this kind of treatment so that by this way the use of unnecessary drugs is avoided, and a considerable part of patients who would not benefit from them are saved from being exposed to toxic and ineffective therapy.

Being a retrospective study with small sample size and absence of correlation against clinico-pathological parameters of the tumour were the main limitations for this study. Moreover, evaluation of other important biomarkers related to mutations in KRAS exon 3 or 4, NRAS, or BRAF were not conducted. Thus we recommend that conduction of larger studies is needed in the future to correlate other variables such as the clinicopathological parameters with the KRAS genotype in Libyan patients with colorectal cancer. More detailed investigation of NRAS, a counterpart of KRAS, and BRAF as downstream signalling effectors, might be of considerable assistance for targeted therapy management.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

Not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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