The Frequency and Spectrum of *K-ras* Mutations among Iraqi Patients with Sporadic Colorectal Carcinoma (CRC)

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ABSTRACT:

BACKGROUND:

CRC is one of the most common cancers in the world. *K-ras* is proto-oncogene with GTPase activity that is lost when the gene is mutated. Analysis of *K-ras* mutational status is very important for CRC treatment, being the most important predictors of resistance to targeted therapy.

OBJECTIVE:

This study aims to determine the frequency and spectrum of *K-ras* mutation among Iraqi patients with sporadic CRC.

PATIENTS, MATERIALS AND METHODS:

This study enrolled 35 cases with sporadic CRC; their clinicopathological parameters were analyzed. The FFPE blocks were used for DNA extraction; PCR amplification of *K-ras* gene and hybridization of allele-specific oligoprobes were performed. The assay covers 29 mutations in the *K-ras* gene (codons 12, 13, 59, 60, 61, 117 and 146).

RESULTS:

The majority of cases have left colonic tumours (57%), without LN involvement (57.1%), of non-mucinous adenocarcinoma histology (85.7%), grade II (82.9%) and stage III (37.1%) tumours. Fourteen mutations were detected in 13 (37%) patients with *K-ras* mutations; 10 (71.4%) mutations were in codon 12 while 4(28.6%) were in codon 13. The most frequent mutation was the G>T transversions [9 (64.4%)] and the most frequent mutation type was GGT>TGT (GLY>CYS) at codon 12.

CONCLUSION:

The incidence of *K-ras* mutations lies in the middle of the reported figures worldwide; the majority of mutations occurred at codon 12 followed by codon 13; predominantly of G>T transversion and Gly12Cys type that has a poorer prognosis.

KEYWORDS: CRC, *K-ras*, codon 12, codon 13.

INTRODUCTION:

Colorectal carcinoma (CRC) is the fifth leading cause of cancer death in both sexes [(1)]. The incidence of CRC in Iraq was 5.36% [(2)]. The majority of CRCs are sporadic (70-80%) with the age being the most important risk factor (3). CRC genetic alterations are basically attributed to genomic instability, which can operate through chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) [(4)]. CIN Pathway accounts for 85% of all CRCs and 65–70% of sporadic CRC. This pathway includes the activation of proto-oncogenes by mutation, including *K-ras*, and inactivation of three tumor suppressor genes: *APC*, *TP53* gene, and subsequent loss of heterozygosity of chromosome 18q [(5)]. *K-ras* mutations constitute about 85% of all of the *Ras* mutations in human tumors [(6)]. Different clinical trials have indicated that a positive effect of anti-EGFR blockage is restricted to patients with *Ras* wild type tumors [(4)].

AIMS OF THE STUDY:

Determine the frequency and spectrum of *K-ras* mutations among Iraqi patients with sporadic CRC using an extended spectrum assay kit and to study the clinicopathological characteristics of sporadic CRC with *K-ras* status.

MATERIALS AND METHODS:

Forty-four surgically resected CRC patients visiting the Gastroenterology and Hepatology Teaching Hospital – Baghdad / Iraq during the

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period extending from Jan. 2014 till Dec. 2015; 9 cases were excluded as they 2 cases have FAP, 1 with IBD, 3 were on chemotherapy / radiotherapy and in 3 cases, the FFPE blocks were missing. From the remaining 35 patients, demographic characteristics as well as tumor topography (including location, size, growth pattern, invasion, lymph node involvement, histological appearance, grading and TNM staging according to the American Joint Committee on Cancer (AJCC) were all reported. The study was approved by the ethical committee of the Ministry of Health / Baghdad – Iraq.

The FFPE blocks from enrolled patients were examined. The slides were examined by two different histopathologists; new slices from the blocks were taken for DNA extraction from areas with less mesenchymal tissue and no necrosis. DNA was extracted from FFPE blocks of all patients using the QIAamp DNA FFPE Tissue Kit (QIAGEN / Germany). The *K-ras* mutation detection was performed using *K-ras* XL StripAssay kit, (Vienna Lab Diagnostics GmbH, Austria). The specified DNA fragment was amplified by a conventional thermal cycler (PCR) using specific biotinylated primers; the amplification program was: Pre-PCR: 37°C/10 min, initial denaturation: 94°C/2 min, following by 35 cycles of 94°C/1 min.; 70°C/50 sec.; 56°C/50 sec.; 60°C/1 min, then by final extension: 60°C/3 min. The amplification products were hybridized to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines on a test strip. The bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates according to the manufacturers' instructions. The assay was designed to detect 29 different *K-ras* mutations in codons (12, 13, 59, 60, 61, 117, and 146). Chi – square test was used for statistical analysis; a p-value <0.05 was considered as statistically significant.

RESULTS:

The ages of the 35 enrolled cases ranged between (20-70) years with a mean±SD of 52.7±13.5 years. Out of the total 35 CRC patients 27(77%) were ≥45 years old and 8(23%) were <45 years. They included 12(34%) males and 23(66%) females (M: F ratio of 1:2). Patients' and tumors' characteristics as well as *K-ras* status of the enrolled cases are illustrated in Table (1).

The majority of cases have left colonic tumours (57%), without LN involvement (57.1%), the tumours had non-mucinous adenocarcinoma histology (85.7%), of grade II (82.9%) and stage III (37.1%).

Out of the 35 enrolled patients, 13(37%) had *K-ras* mutations. A total of 14 mutations were detected in the tumors of the 13 patients [12/13 cases had a single mutation, while 1/13 had double mutations (one in codon 12 and the other in codon 13)]. Of these mutations 10 (71.4%) were in codon 12 while 4(28.6%) were in codon 13; mutations in other codons (i.e. 59, 60, 61, 117 and 146) were not reported in this study. The most frequently encountered mutations were G>T transversions, which were found in 9 (64.4%) cases, while the remaining 5 (35.6%) cases had G>A transitions.

Four types of mutations were detected in this study; the most frequent was GGT>TGT (GLY>CYS) at codon 12 as seen in 8 (57.2%) cases, followed by GGC>GAC (Gly>Asp) at codon 13 seen in 3(21.4%) cases; the remaining mutations were as follows: GGT>GAT (GLY>ASP) at codon 12 constituting 2 (14.2%) cases and GGC>TGC (Gly>Cys) at codon 13 constituting 1 (7.2%) cases; Table (2).

There was no statistically significant association between each clinicopathological characteristic (age, gender, tumor location, histological type, LN involvement, grade and TNM stage) with *K-ras* mutation status, p-value was >0.05; Table (1).

Table 1: Patients' characteristics, tumor characteristics and *K-ras* status of 35 Iraqi patients with sporadic colorectal carcinoma.

Parameter	Number (%)			p-value
	Total	Patients with Wild-type *K-ras*	Patients with mutant *K-ras*	
Number of cases	35	22(63%)	13(37%)	
Age				
Mean±SD	52.7±13.5	53.9±14.0	50.6±12.8	0.527
Gender				0.463
Male	12(34%)	9(40.9%)	3(23.1%)	
Female	23(66%)	13(59.1%)	10(76.9%)	
Tumor location				0.175
Right colon	7(20%)	6(27.3%)	1(7.7%)	
Left colon	20(57%)	10(45.5%)	10(76.9%)	
Rectum	8(23%)	6(27.3%)	2(15.4%)	
L.N involvement	15(42.9%)	10(45.5%)	5(38.5%)	0.737
Histology				0.886
Mucinous	5(14.3%)	3(13.6%)	2(15.4%)	
Non - mucinous	30(85.7%)	19(86.4%)	11(84.6%)	
Grade				0.818
GI	4(11.4%)	3(13.6%)	1(7.7%)	
GII	29(82.9%)	18(81.8%)	11(84.6%)	
GIII	2(5.7%)	1(4.5%)	1(7.7%)	
Stage				0.846
I	11(31.4%)	7(31.8%)	4(30.8%)	
II	8(22.9%)	4(18.2%)	4(30.8%)	
III	13(37.1%)	9(40.9%)	4(30.8%)	
IV	3(8.6%)	2(9.1%)	1(7.7%)	

Table 2: The spectrum of *K-ras* mutations at codon 12 and 13 detected among 13 Iraqi patients with sporadic CRC.

Codon	Wild-type	Mutant	Mutation	Amino Acid	No.	%
12	GGT	TGT GAT	G>T G>A	GLY>CYS GLY>ASP	8 2	57.2% 14.2%
13	GGC	GAC TGC	G>A G>T	GLY>ASP GLY>CYS	3 1	21.4% 7.2%
Total					14	100%

DISCUSSION:

K-ras mutation status among patients with sporadic CRC is one important parameter determining the usefulness of anti-EGFR therapy, as *K-ras* mutations lead to resistance to targeted therapy and have been associated with poorer survival and increased tumor aggressiveness [7]. The presence of activating *K-ras* mutations has been identified as a potent predictor of resistance to EGFR-directed antibodies such as cetuximab or panitumumab [8].

This study is one of few, probably the only, Iraqi study analyzing *K-ras* gene for an extended spectrum of mutations (29 mutations in 7 codons) among sporadic CRC patients.

The majority [27 (77%)] of patients in the current study were older than 45 years, consistent with the multistep genetic process in colorectal carcinogenesis, requiring a long period of time for multiple genes to undergo mutation by the effect of environmental carcinogens on colonic

mucosa [9]. These findings were in agreement with many local and regional studies [10,11,12].

Worldwide, the incidence of CRC is higher in men [13] as estrogen plays an important protective role in the pathogenesis of colorectal carcinoma in premenopausal women [14], although many studies showed contrasting data, ranging from male predominance, through equal M: F ratio to female predominance [15,16,17]. Comparable levels of CRC risk are reached in women as compared to men at a higher age (18). In the present study there was female predominance as most cases were older age group.

K-ras mutation rates reported worldwide varies widely from 13% to 60% [19,20], with heterogeneity in mutation rates among different ethnicities due to different genetic backgrounds, environmental and lifestyle differences between the nations [21].

The overall observed frequency of *K-ras* mutations in this study was 37%. Other local studies showed almost similar incidence of 38.5% and 48% respectively [22,23]. Regional studies from Iran & Turkey also showed similar findings [24,25].

The reported incidence lies between that of Asian / Middle Eastern cases (24%) and that of European / Latin American cases (36% and 40%) [21].

The predominant mutation observed in the current study was G>T transversion, occurring in 9(64.4%) cases, while G>A transition was detected in 5 (35.6%); Table (2). A previous Iraqi study revealed that the G>T transversions and the G>A transitions were equal in frequency and constituted 41.4% each [23], whereas the frequency of G>T transversion was (49%) and G>A transition was 47% in Iran [24].

In spite of the racial and geographic variations, our results were nearly similar to that reported from a similar study in Yugoslavia in which G>T transversions make up 77% of all mutations present [26]. These findings contrast many studies from Turkey, Jordan and Saudi Arabia [27,28,29], where G>A transitions far exceeded G>T transversions. Such diversity could be explained by considering geographical, environmental differences and dietary habits as G>A transitions are the characteristic effects of alkylating agents such as N-nitroso compounds produced in red and processed meat [30], whereas G>T transversions are considered to be induced by carcinogenic agents like polycyclic aromatic hydrocarbons found in dietary fats [31], ionizing radiation or carcinogens such as those found in cigarette smoke [32]. This might explain its higher levels of G>T transversion in our cases as Iraq passed through >35 years of wars and progressive environmental pollution and increasing smoking habits in recent history [33].

The *K-ras* mutations in CRC cases were located in exon 2 (codon 12 and 13) in more than 90% of cases, while the rest are found mainly in exon 3 (4% at codon 61) and exon 4(1–2% at codons 117 and 146) [34]. In this study, codon 12 was found to be the major culprit of the event, contributing to 10 (71.4%) of total detected mutations followed by codon 13 with 4(28.6%); Table (2); this figure was consistent with other local and worldwide studies [19,22,23,28,35-37].

The biological and functional consequences of *K-ras* mutations at codon 12 may be different from those at codon 13 as tumors harboring codon 13 *K-ras* mutations may benefit from anti-EFGR monoclonal antibodies therapy [38].

Mutations at codons other than codon 12 and 13 were not detected in this study, which is similar to [35,39] from Iran; these mutations seem to be much rarer than codon 12 or 13 and need larger sample size to appear.

Multiple mutations are not uncommon in CRC cases. Their presence is associated with a more aggressive tumour behavior [40]. Macedo et al. (2011) identified 69 cases of multiple mutations in *K-ras* [41]. In the current study, multiple *K-ras* mutations were detected in one case only occurring at codon 12 and 13 simultaneously; multiple *K-ras* mutations were also detected in another Iraqi study [23] and other studies from different countries [40,42-43].

CRCs harboring the Gly12Cys or Gly13Asp mutations were significantly associated with worse prognosis [44]. In the current study, Gly12Cys (GGT>TGT) was found to be the most frequent mutation type accounting for 8(57.2%) of all detected mutations; Table (2). Based on these data, Iraqi patients might have a poorer prognosis. This needs larger case studies to prove/disprove this observation.

Recent evidence associates the Gly12Val mutant K-ras protein with higher stage and increased lethality of CRC, while the Gly12Asp mutation showed no such association; it was proposed that Gly12Val mutant *Ras* generates a more persistent, potentially oncogenic signal as compared to Gly12Asp mutant or wild-type *Ras*

protein [45]. Gly12Val mutant *K-ras* was not reported in our cases, but was reported in an earlier Iraqi study being the most frequently detected mutation type [23].

Gly12Ala (GGT>GCT) was the most frequent mutation type detected in Southern Iran [42]; these results contrast other regional studies [28-29,36] that showed predominance of Gly12Asp (GGT>GAT); the frequency of *K-ras* mutations in different studies could be similar but the mutational spectrum could vary and is influenced by genetic and environmental factors [24].

Codon 13 mutation Gly13Asp (GGC>GAC) was the predominant type in the current study, which accounts for 3(21.4%) of total detected mutations; Table (2), which was consistent with other studies [19,23,28,36].

The association of *K-ras* mutation with tumor stage is controversial; some studies reported statistically significant association of *K-ras* mutation with advanced tumor stage [22,29].

On other hand, many studies reported no such significant association, including this study and other local and worldwide studies [23,27-28, 37,46].

It is observed in our study that 12(92.3%) of tumors harboring *K-ras* mutations were without distant metastasis (stage I, II and III) i.e M0 stage and only 1(7.7%) presented with distant metastasis (stage IV) i.e. M1 stage; Table (1). This supports the hypothesis that *K-ras* mutations are mostly an early event in the process of CRC tumorogenesis [47].

CONCLUSION:

K-ras mutations are relatively common among Iraqi patients with sporadic CRC. The frequency of *K-ras* mutations among sporadic CRC patients lies in the middle of the reported figure worldwide, somewhere between the Asian / Middle Eastern and the European / Latin American figures; these tumors might have a poorer prognosis, having predominance of codon 12 *K-ras* mutations and Gly12Cys type with predominance of G>T transversion.

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Competing interests

The authors declare that they have no competing interests.

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Author contribution:

Shaymaa KS Al-Thahir contributed to the collection of samples, performing the major part of molecular diagnosis, interpretation of results and drafting of the manuscript.

Bassam MS Al-Musawi contributed to the concept and design of the work, performing part of the molecular work, interpretation of results and drafting of the manuscript.

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