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Detection of polymorphism *Kras* gene in colorectal cancer (CRC) of Iraq patient

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Abstract

Mutation in the Kirsten Ras (KRAS) oncogene in colorectal cancer (CRC), the role of KRAS mutation status as a prognostic factor. The aim of study between KRAS mutation status and CRC survival. We evaluated KRAS mutations in codon 12, 13 and 61 of exon 1,2 and 3 of KRAS gene by polymerase chain reaction (PCR) and reverse-hybridization of 74 patients were male 42 (56.76%) and female 32 (43.24%), the patients ages ranged from 25 to 80 (median 52.5). The results appeared three transversion mutation (34 G>T, 35 G>T, and 35 G>T) that caused change amino acid from glycine (GGT) to Cysteine (TGT), aspartic acid (GAT) and valine (GTT) respectively, the proportion of94.94% from all sample compared with control samples. And percentage 5.06% appeared transition mutation at position 38G>A of codon 13 of exon 2 of the KRAS gene that caused change amino acid from glycine (GGC) to aspartic acid (GAC), however, no mutation at codon 61 of exon 3 KRAS gene. The conclusion: mutation in KRAS codon 12 of exon 1 are associated with colorectal cancer in Iraq patients.

Keyword: Colorectal cancer, Polymorphism, KRAS gene, Transversion, Transition, Iraq.

Introduction

Colorectal cancer (CRC) is one at the leading causes of deaths in western countries including the United States CRC was reported to be responsible for 9% of new cancer cases and 10% of cancer deaths in 2010 in the United States alone (Hassen *et al.*, 2012). CRC is a disease originating from the epithelial cells of colon or rectum, it is most frequently result of mutations that may be inherited or acquired (Markowitz, 2009). The development of colorectal cancer is a multi-step process characterized by the accumulation of genetic alterations (Arteaga, 2002; Russo *et al.*, 2009). One of various genetic alterations, an important molecular target for metastatic CRC treatment is the epidermal growth factor receptor (EGFR) (Arteaga, 2003). The KRAS (Ki-ras2) Kirsten rat sarcoma represent an early event in development and progression of CRC because there is substantial evidence that K-RAS point mutations predict resistance to epidermal growth factor receptor(EGFR) directed therapy of metastatic colon cancer has been presented recently (Allerga *et al.*, 2009).The K-RAS protein is part of the pathway linking epidermal growth factor signaling to activation of proliferation-related genes in the nucleus (Siena *et al.*, 2009). Hence, oncogene mutation in the K-RAS gene result in a constitutively active protein, causing growth factor-independent stimulation of the downstream pathway and resistance against therapy that targets EGFR (Siena *et al.*, 2009). The K-RAS protein is involved in transduction of mutagenic signals RAS/MAPK signaling pathway leading to proliferation, differentiation and apoptosis. The Ras protein is activated transiently as a response to extra-cellular signal such growth factor, cytokines and hormones that stimulate cell surface receptors (Karreth and Tuveson, 2009). Oncogen K-RAS mutations which prevent the hydrolysis of GTP, are found in a variety of human malignancies, as single events mostly occurring at codon 12 or 13 in exon 2 (Breivik *et al.*, 2005). In colorectal cancer (CRC) patients, they account for more than 90% of the occurring K-RAS mutation, and confer resistance therapeutic antibodies that block the EGF receptor. This evidence supports the importance of K-RAS gene mutational status as molecular predictive determinant for the biological therapy of colorectal cancer (Normanno *et al.*, 2009). The aim of this work was to study the polymorphism of *Kras* gene in colorectal cancer (CRC) patients in Baghdad, Iraq.

Materials and Methods

Samples and DNA extraction: Biopsies (Fresh tissue)

samples were obtained from 74 Iraqi patients affected by colorectal cancer (age averaged 52.5 years old from 25 to 80 years) and also obtained 74 samples from patient with bowel inflammation which used as control samples. Patients were admitted to the Imamain Al- Kadhymain Medical City, Gastroenterology and Hematology Diseases Center and Baghdad teaching hospital. The disease was clinically diagnosed by the consultant medical staff at the centre.Fresh samples of tumor tissues were examined by a physician and were kept at -20°C until extraction of DNA was performed. In total, three pieces of tissue tacking by biopsy were used to extracted DNA which weighing no more than 25mg of tissue. DNA was extracted from tissue by DNA extraction kit (QIAamp DNA Mini Kit, Qiagen, Germany) and DNA extraction from formalin fixed, paraffin-embedded (FFPE) tissues by QI-Aamp DNA FFPE Tissue Kit according to the manufacturer's protocol.

Detection of KRAS gene mutation: Assay for the identification of KRAS mutations based on polymerase chain reaction (PCR) and reverse-hybridization. The assay covers 10 mutations in the KRAS gene (codon 12 and 13). The procedure includes three steps: (1) DNA isolation, (2) PCR amplification using biotinylated primers, (3) hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines (Figure 1). Bound biotinylated sequences are detected using streptavidin-alkaline phosphatase and color substrates.

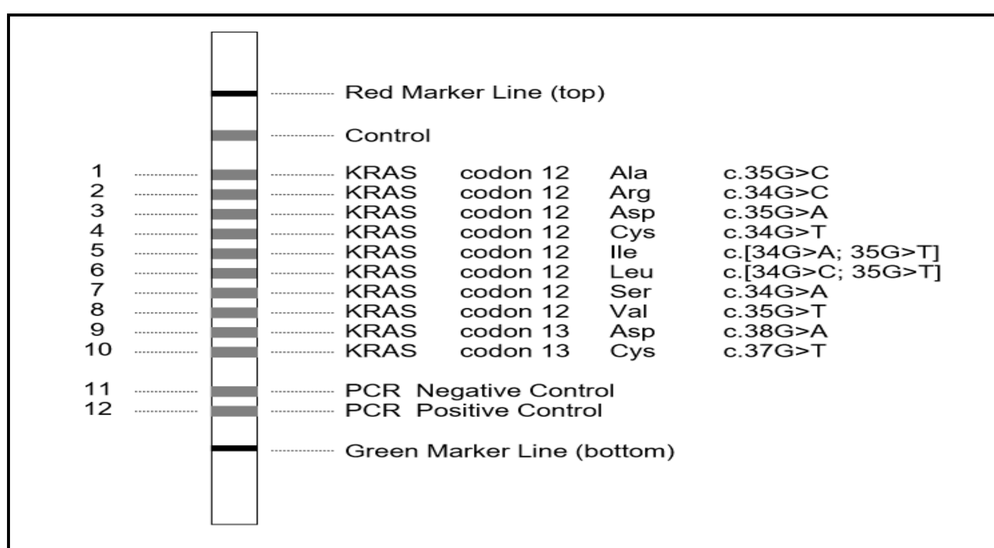

Figure (1): Teststrip design

Each sample had prepare in a 25µl final PCR reaction mix: 15µl from Amplification Mix (yellow cap), 5µl diluted (1:25, final conc. 0.2U/µl) Taq DNA polymerase (1U) and 5 µl of template DNA. The thermal cycling conditions were done as follows: Pre-PCR: 37°C for 10min. and 94°C for 2min. Thermo cycling: 94°C for 1min, 70°C for 50sec, 56°C for 50sec, 60°C for 1min repeated for 35 cycles. Final extension: 60°C for 3min.using a thermal cycler (Gene Amp, PCR system 9700; Applied Biosystem). Ten microliter of DNAT (blue cap) was pipetted into the lower corner of each lane to be used in the Typing Trays (one lane per sample). Ten microliter of amplification product (PCR) was added into the corresponding drop of DNAT, mixed thoroughly with a pipette and incubated for 5min at room temperature. Than one milliliter hybridization buffer was added (prewarmed to 45°C before used), into each lane. Teststrips had been inserted into the respective lanes (Submerge completely). Tray had incubated for 30min at 45°C on the shaking platform of the waterbath. At the end of incubation hybridization solutions had remove by pipetting. One milliliter of wash solution A was added, then rinse briefly for 10sec., liquids was remove pipetting. And one milliliter of wash solution A was added and incubate for 15min at 45°C in the shaking waterbath, (This step was repeated twice). Then one milliliter from conjugate solution was added and incubate for 15min at room temperature with shaking, and this step was repeated for 10 sec. Liquids was removed and one milliliter of wash solution B was added and incubates for 5min at room temperature with shaking, after liquid was removed, washing was repeat again. And one milliliter of color developer was added and incubated for 15min at room temperature in the dark with shaker than a purple staining will appear

upon positive reaction. Strip was reading compared with reference.

Results and Discussion

KRAS gene was successfully amplified using biotinylated primers for codon12, 13 of exon 1 and 61 of exon 2. Figure (2) showed PCR amplification of codon 12 exon 1 of the *KRAS* mutations at positions (34 G>T, 35 G>T, 34 G>C, 34 G>C, {34 G>A; 35 G>T}, {34 G>A; 35 G>T}, and 35 (G>T)), which related to development of disease.

The results appeared three transversion mutation (34 G>T, 35 G>T, and 35 G>T) that caused change amino acid from glycine (GGT) to cysteine (TGT), aspartic acid (GAT) and valine (GTT) respectively and showed that 31 sample from 74 of colorectal cancer (CRC) patient have 12,8, and 11 mutations respectively, the proportion of 94.94% from all sample compared with control samples. And Percentage 5.06% appeared transition mutation at position 38G>A of codon 13 of exon 2 of the KRAS gene that caused change amino acid from glycine (GGC) to aspartic acid (GAC), however, no mutation at codon 61of exon 3 KRAS gene is shown in Figure (3).

In this study, distribution of patients (74 tumors) into wild type (55.41%) and mutant KRAS (44.59%), in codon 12 and 13 did show significant between two groups (P<0.05).

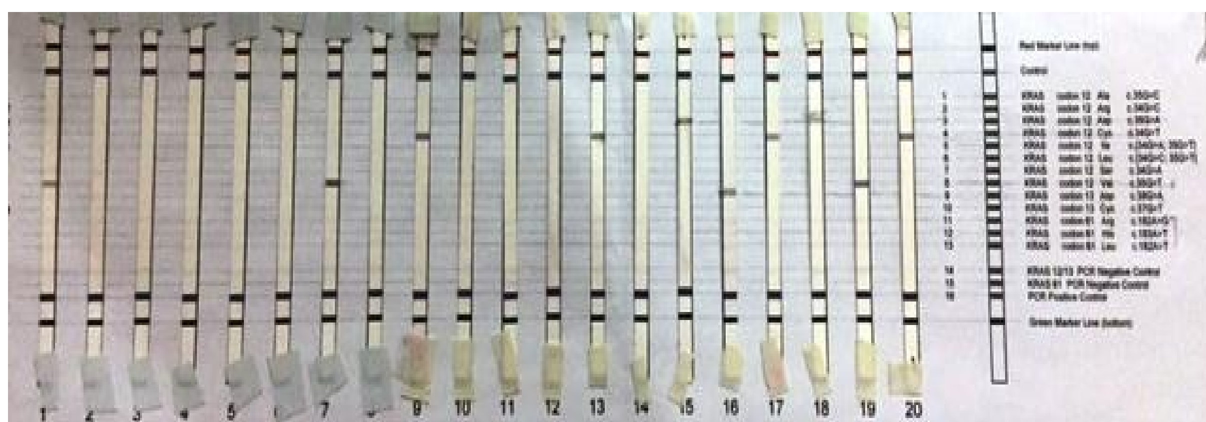

Figure (2): Teststrip design shown strip number 1, 7, 19 represent mutation (Val 35 G>T) at codon 12, strip number 16 represent mutation (Asp 38G>A) at codon 13 , strip number 9, 13, 17, 20 represent mutation (Cys 34 G>T), strip number 15, 18 represent mutation (Asp 35G>A) at codon 13, strip number 2, 3, 4,5,6,8,10,11,12, 14, 16, represent no mutation.

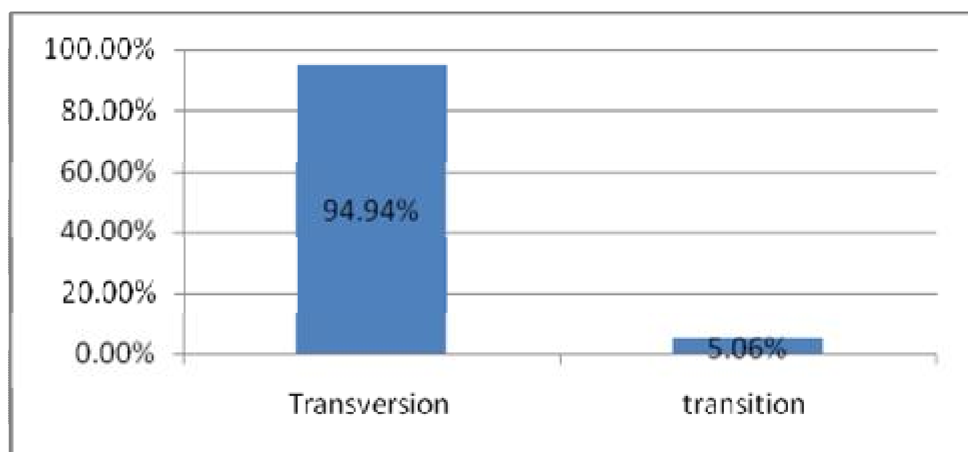

Figure (3): Mutation events in KRAS gene. KRAS codon 12 and codon 13 analysis in 74 patients.

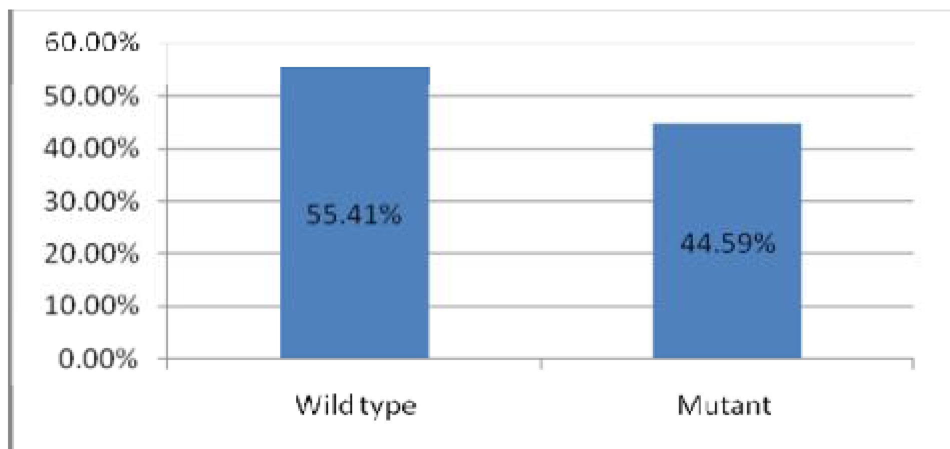

Figure (4) Frequency of KRAS mutations among Iraqi CRC patients.

KRAS mutations did not show any significant correlation with tumor location, and gender of the patient, shown Figure (5) and Table (1), the percentage of its frequency in female 48.48%and male 51.52%, and the incidence of KRAS mutation was similar in men and women in 159 coloretecal cancer Albanian patients (Martinetti *et al.,* 2014). And equal numbers of male and female have KRAS mutation in coloretecal cancer of Jordanian patients (Elbjeirami and Sughayer, 2012), this corresponds with Ferreira *et al.* (2010) and Nagasaka *et al.* (2004) have reported a higher frequency of KRAS mutations in females compared with males. However, this different from other research indicated that KRAS mutation occurred more frequency in females than in male (P>0.02) (Kadowaki *et al.*, 2015). KRAS mutations (Codon 12 and 13) did not show any significant correlation with differentiation colon, and age at onset of the patient, this compatibility with other result no association between KRAS mutations such as age, tumor differentiation, UICC classification in colon cancer from Albanian patients (Martinetti *et al.,* 2014).

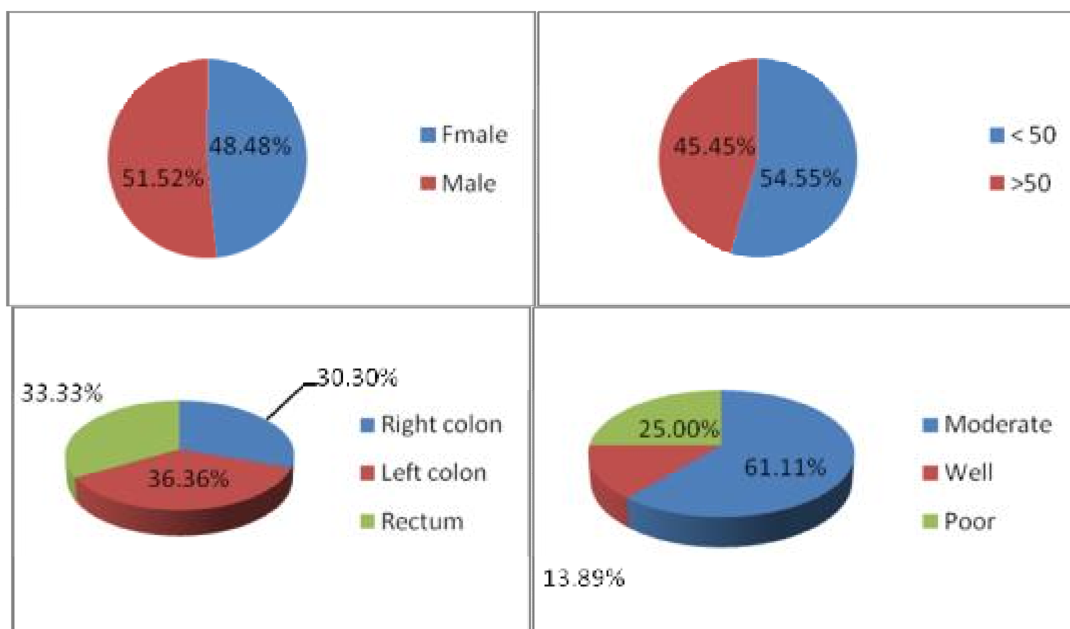

Figure (5): Effect of gender, age, location colon, differentiation colon percentage of *KRAS gene.*

Table (1): Effect of age, gender and site in number and percentage of *KRAS*

Characteristic		Total no (%)	KRAS (+)	KRAS (-)
		74	33	41
Age \ years	< 50	32 (43.24%)	18 (54.55%)	14 (34.15%)
	>50	42 (56.76%)	15 (45.45%)	27 (65.85%)
	Chi-square	5.109 *	4.792 *	10.337 **
	P-value	0.0478	0.0492	0.0026
Gender	Male	42 (56.76%)	17 (51.52%)	25 (60.98%)
	Female	32 (43.24%)	16 (48.48%)	16 (39.02%)
	Chi-square	5.109 *	0.861 NS	9.025 **
	P-value	0.0478	0.633	0.00319
Site	Right colon	19 (25.68%)	10 (30.30%)	9 (21.95%)
	Left colon	29 (39.19%)	12 (36.36%)	17 (41.46%)
	Rectum	26 (35.14%)	11 (33.33%)	15 (36.59%)
	Chi-square	10.437 **	2.075 NS	9.244 **
	P-value	0.00419	0.0968	0.00762
Differentiation	Moderate	48 (64.86%)	22 (61.11%)	26 (68.42%)
	Well	6 (8.11%)	5 (13.89%)	1 (2.63%)
	poor	20 (27.03%)	9 (25.00%)	11 (28.95%)
	Chi-square	12.448 **	10.957 **	12.944 **
	P-value	0.0001	0.0026	0.0001

* (P<0.05), ** (P<0.01).

This study investigated the general incidence of KRAS mutations in colorectal cancer (CRC) patient in Iraq and the incidence of specific mutation types, PCR was performed to amplify codon 12 and 13 in KRAS gene. Elbjeirami and Sughayer (2012) referred to 39 mutation in codon 12, distributions to 62.5% were transition mutation (GGT>GAT), however in codon 13 were present only GAC transition mutation. In most countries, the KRAS mutation rate from 18% as identified in Egypt (Andreyev, *et al.*, 2001). However high rate of KRAS mutation in United States about 47%, this range higher than the fraction mutations in codon 12 and 13 of Saudi Arabia and Turkey (28 and 37.5% respectively) (Abubaker *et al.*, 2009). And referred many of research a significant number about 44% of KRAS mutations were detected using their screen Dxs KRAS mutation kit for a large number of mutation in Jordanian population. Brink *et al.*, 2003 referred to 37% of the patients , the exon 1 fragment of KRAS gene was found to be mutated and study scanning codon from 8- 29, and showed mutations are G>A transitions and G>T trans version and codon 12, 13 are the most frequently affected codons.

The many studies referred to point mutations in KRAS on cogene have been investigated in colorectal cancer (Baisse *et al.*, 2001). Kislitsin *et al.* (2000) found 90% of the activating mutations are found in codon 12 (wild type GGT) and codon 13 (wild type GGC) of exon 1 and 2 respectively and 5% in codon 61 (wild type CAA) located in exon 2. Inoue *et al.* (2012) refer to role mutations in KRAS are evident in 30-40 % of colorectal tumors. Phipps *et al.* (2013) referred to KRAS mutated CRC was associated with statistically significantly poorer survival after diagnosis than KRAS wild type CRC. KRAS mutations are almost single nucleotide point mutation as reported and the most common patterns are G12D, G12A, G12R, G12C, G12V, and G13D. In the codon 12 mutation PG12D, PG12V, is the most frequent and in codon 13, the substitution of glycine for aspartate (G13D) in the most frequent (Neumann *et al.*, 2009)

The study compatibility with other study, Van *et al.* (2011) and Karapetis *et al.* (2008) referred to mutant KRAS is found in about 35-45% of CRC and codon 12 and 13 which account for about 95% of all mutation types with approximately 80% in codon 12 and 15 % in codon 13. However, Forbes, *et al.* (2006) referred to other mutations in codon 61, 146, 154 occur less frequently for 5% of all mutations.

Arrington *et al.* (2012) showed KRAS mutations in colon cancer have been associated with poorer survival and increased tumor aggressiveness. Chen *et al.* (2014) our findings indicate that KRAS mutation were identified in 44.9% (96 patient from 214 sample) in the colorectal cancer CRC patient from western countries. Numerous studies have indeed observed that KRAS mutations are commonly present in CRC with frequencies of 30-50%, KRAS mutation occur 90% in codon 12 and 13 (Roock *et al.*, 2011). Zulhabri (2012) found single base transition from GGT to GAT (glycine to

aspartate) in codon 12 was detected in nine samples while three samples presented with GGC to GAC transition in codon 13 and showed a high frequency of G to A transition of codon 12 mutation of the K-ras gene with significant correlation with tumor size and tumor location.

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