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Research paper

New Finding in KRAS Mutated Structure Detected in Colorectal Cancer CRC Tumors Causing Uncontrolled Cell Proliferation in Iraqi Patients

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Abstract

A total 200 tumor samples were collected from patients suffering from colorectal CRC at Al-Amal hospital. Their ages ranged from 40 – 70 years old and distributed according to gender as 130 male and 70 female. We found that CRC was more common in male than female, and more frequent in elderly persons especially in age group of 61 – 70 years old. From 12 specifically designed primers that were able to amplify KRAS gene in those patients' blood samples, only 2 gave a specific band when tumor sample was used. Molecular analysis of the sequence obtained from those two primers showed the presence of 83 missense mutations in both of them, 20 in the first sequence were associated with pathogenic effect on KRAS gene, 3 frame shift, and 3 insertions, while in the second sequence obtained from primer PK2 4 frame shift mutations, and 4 insertions were identified. The impact finding is that in both sequences, open reading frames (ORFs) were developed that affected cell proliferation dramatically and produced truncated functionless proteins causing KRAS to cease control over cell cycle.

Keywords: *KRAS; Oncogene; colorectal cancer; pathogenetic mutation.*

1. Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related death. The development of CRC is a multistep process characterized by accumulation of genetic alterations that have long been considered to occur in a stepwise process (Russo *et al.*, 2005. Along the progression from normal colonic epithelial cells, small adenoma, advanced adenoma, and finally to carcinoma, the KRAS oncogene mutation has a role in a significant proportion of CRCs Castagnola and Giaretti 2005. KRAS has been reported to be mutated in about 30% of colorectal adenomas and 30% to 50% of CRCs. The KRAS gene encodes a 21-kDa small protein that is activated transiently as a response to extracellular stimuli or signals such as growth factors, cytokines, and hormones via cell surface receptors. On its activation, the KRAS protein also is capable of turning off the signaling pathway by catalyzing hydrolysis of guanosine triphosphates (GTP) to guanosine diphosphates Downward 2003. The most common KRAS mutations in codons 12 and 13 are activation mutations, leading to continuous activation of downstream pathways. The most frequently observed types of mutations in KRAS in all human cancers are G > A transition and G > T transversion Haigis *et al.*, 2008. Although the precise molecular and cellular mechanisms that constitute the oncogenic effects of activating KRAS mutations remain incompletely understood, in vitro and animal studies showed that KRAS regulated genes involve cytokine signaling, cell adhesion, cell survival, proliferation, apoptosis, and colon development Malumbres and Barbacid, 2003. The RAS oncogene has a well-established role in cell growth and regulation; and its protein product affects many cellular functions including cell proliferation, apoptosis, migration, fate specification, and differentiation. There are three known human isoforms, NRAS, HRAS, and KRAS. Over 90% of pancreatic adenocarcinomas Almoguera , 1988 , 30%–50% of colorectal cancers De Roock et al, 2008, 55% of thyroid cancers, 35% of lung cancers, and 35% of rhabdomyosarcomas harbor mutated RAS genes Kranenburg 2005. Although HRAS was historically the most studied RAS gene, it is actually the isoform least mutated in human cancers Baines, and Xu, D.; Der 2011. In fact, KRAS mutations comprise 86% of all RAS mutations. Mutations in KRAS occur with the greatest frequency in all human cancers (21.6%), followed by NRAS (8.0%), and HRAS (3.3%). In recent studies, a lethal genetic change was identified at exon 2 in KRAS gene in Iraq. The study showed the accumulation of 22 pathogenic SNPs which eventually led to CRC in these patients (Subhi *et al.*, 2018).

2. Materials and Methods

Collection of tissue Samples: Tissue samples were collected at Al-Amal hospital from 200 patients after they diagnosed with colorectal cancer using bio marker (CA19-9) and subjected to tumor excision. Their ages ranged between (40-70) years as the majority of individuals attending the hospital for treatment.
DNA extraction from blood: The gSYNC™ DNA Extraction Kit from Geneaid (Taiwan) was used for this purpose as instructed by the manufacturer. The extraction procedure mainly depends upon spin column technique which gave DNA purity of 1.8-2 and concentration of 80-120 ng/sample. Primers used for DNA amplifica-

tion: KRAS exon2 was amplified using the following primers designed in this study.

Primer name		Sequences 3`---- 5`	Product size bp	Reference
PK1	F	GTCTCCCTGTGTCAGACTGC	433	Designed in this study*
	R	AATGTCTTGGCACACCACCA		
PK2	F	CTTCCACATGCCCCATGACT	370	Designed in this study*
	R	ACTGTTACCAGGAGCAGTCC		

DNA Amplification Programs and PCR Conditions

Because of the different melting temperatures for each set of primer, the following conditions were used for optimum results:

Primers PK1: Initial Denaturation 1 cycle 94°C for 5 min followed by 35cycles of Denaturation 94°C for 1 min Annealing 59°C for 1 min Extension 72°C for 1 min, after that Final extension 1 cycle 72°C for 10 min and kept at 40C until stored at -200C.

Primer PK2: Initial Denaturation 1 cycle 94°C for 5 min followed by 35cycles of Denaturation 94°C for 1 min Annealing 56°C for 1 min Extension 72°C for 1 min Final Extension 1 cycle 72°C for 10 min, and kept at 40C until stored at -200C.

Electrophoresis Conditions: The resulting PCR products were subjected to electrophoresis using 2% agarose at 10 v/cm field strength for 1 h and photographed using Biorad gel documentation system.

3. Result and Discussion

Clinicopathologic Features of CRCs in the Study Population KRAS is perhaps best characterized in colorectal cancer. In 1988, Vogelstein *et al.* first proposed a model for a sequence of genetic events leading to the development of colorectal cancer. Point mutations in KRAS were described as an early event in the pathogenesis of colorectal cancer. In fact, KRAS mutations were demonstrated in 50% of adenomas and described as a key genetic alteration necessary for the progression of adenoma to colorectal cancer. Thus, many have hypothesized that development of KRAS mutation is an important role in the multi-step process early in carcinogenesis Amanda *et al.*, 2012. Among 12 designed primers specifically to amplify exon2 of KRAS gene, only two were able to give specific band from CRC tissue. Sequence blast of these bands showed similarity with 98% to the exon under study.

Mutational Types of KRAS in the Study Population: In this study, among the 200 cases with analyzable KRAS results, 150 tumors (75%) were found to harbor mutated KRAS gene. The KRAS mutations were distributed between codon 47020 and codon 47400 which showed 83 missense mutations with 20 pathogenic once, 4 frame shift mutations, 4 insertions, and 2 deletions as showen in table (1).

Table 1: Types and locations of mutations identified at position 47020 – 47400 of chromosome 12, KRAS gene.

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
25209808	746	rs775000854		Missense	A	Tyr [Y]	2	185
				contig reference	G	Cys [C]	2	185
25209822	730	rs749587181			-		1	180
25209822	732	rs762532538		Missense	C	Asn [N]	3	180
				contig reference	G	Lys [K]	3	180
25209826	728	rs763736188		Missense	G	Arg [R]	2	179
				contig reference	A	Lys [K]	2	179
25209828	725	rs587782954	Uncertain significance		GAA	Lys [K]	2	180
25209829	723	rs397517043	Uncertain significance		-		3	180
25209842	712	rs900508259		Missense	A	Ser [S]	1	174
				contig reference	G	Gly [G]	1	174
25209854	700	rs369501492	other	Missense	T	Leu [L]	1	170
				contig reference	A	Met [M]	1	170
25209871	683	rs372793780	Uncertain significance	Missense	A	Gln [Q]	2	164
				contig reference	G	Arg [R]	2	164
25209884	670	rs757674707		Missense	A	Ile [I]	1	160
				contig reference	G	Val [V]	1	160
25209892	662	rs794727720	Uncertain significance	Missense	G	Cys [C]	2	157
				contig reference	A	Tyr [Y]	2	157
25209894	660	rs104894362	Pathogenic	Missense	G	Leu [L]	3	156
				contig reference	C	Phe [F]	3	156
25209896	658	rs397517042	Pathogenic	Missense	A	Ile [I]	1	156
				Missense	G	Val [V]	1	156
				contig reference	T	Phe [F]	1	156
25209898	656	rs755177746		Missense	G	Gly [G]	2	155
				contig reference	C	Ala [A]	2	155
25209904	650	rs104894360	Pathogenic	Missense	G	Gly [G]	2	153
				Missense	T	Val [V]	2	153
				contig	A	Asp	2	153

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
				reference		[D]		
25209907	647	rs104894367	Pathogenic	Missense	G	Gly [G]	2	152
				contig reference	T	Val [V]	2	152
25209908	646	rs397517041	Likely pathogenic	Missense	T	Phe [F]	1	152
				contig reference	G	Val [V]	1	152
25225625	631	rs387907206	Pathogenic	Missense	G	Glu [E]	1	147
				contig reference	A	Lys [K]	1	147
25225628	628	rs121913527	Uncertain significance	Missense	A	Thr [T]	1	146
				contig reference	G	Ala [A]	1	146
25225641	615	rs138669124		Missense	A	Leu [L]	3	141
				contig reference	T	Phe [F]	3	141
25225651	605	rs754870563		Missense	A	Glu [E]	2	138
				contig reference	G	Gly [G]	2	138
25225652	604	rs778702415		Missense	A	Arg [R]	1	138
				contig reference	G	Gly [G]	1	138
25225657	599	rs757816355		Missense	A	Asn [N]	2	136
				contig reference	G	Ser [S]	2	136
25225663	593	rs373500216		Missense	G	Gly [G]	2	134
				contig reference	C	Ala [A]	2	134
25225675	581	rs730880473	Uncertain significance	Missense	T	Val [V]	2	130
				contig reference	C	Ala [A]	2	130
25225681	575	rs746609817		Missense	G	Arg [R]	2	128
				contig reference	A	Lys [K]	2	128
25225684	572	rs781634879		Missense	G	Arg [R]	2	127
				contig	C	Thr	2	127

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
				reference		[T]		
25225694	562	rs575569675		Missense	T	Ser [S]	1	124
				contig reference	A	Thr [T]	1	124
25225709	547	rs730880471	Likely pathogenic	Missense	A	Asn [N]	1	119
				contig reference	G	Asp [D]	1	119
25225713	543	rs770248150		Missense	C	Asn [N]	3	117
				contig reference	A	Lys [K]	3	117
25225717	539	rs202247812	untested	Missense	G	Ser [S]	2	116
				contig reference	A	Asn [N]	2	116
25225730	526	rs775836436		Missense	A	Ile [I]	1	112
				contig reference	G	Val [V]	1	112
25225742	514	rs763553461		Missense	T	Tyr [Y]	1	108
				contig reference	G	Asp [D]	1	108
25227234	482	rs727503106	Likely pathogenic	Missense	A	Lys [K]	2	97
				contig reference	G	Arg [R]	2	97
25227262	454	rs953088090		Missense	G	Glu [E]	1	88
				contig reference	A	Lys [K]	1	88
25227263	453	rs397517038	Uncertain significance	frame shift	-	Asn [N]	3	88
				contig reference	T	Lys [K]	3	88
25227288	428	rs868857258		Missense	C	Pro [P]	2	79
				contig reference	T	Leu [L]	2	79
25227294	422	rs756890312		Missense	C	Ala [A]	2	77
				contig reference	G	Gly [G]	2	77
25227300	416	rs780974222		Missense	C	Ala [A]	2	75
				contig	G	Gly	2	75

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
				reference		[G]		
25227304	412	rs770020203		Missense	G	Ala [A]	1	74
				contig reference	A	Thr [T]	1	74
25227308	408	rs104886028	untested	Missense	A	Ile [I]	3	72
				contig reference	G	Met [M]	3	72
25227310	406	rs727504662	Pathogenic	Missense	T	Leu [L]	1	72
				contig reference	A	Met [M]	1	72
25227313	403	rs387907205	Likely pathogenic	Missense	C	His [H]	1	71
				Missense	C	His [H]	1	71
				Missense	G	Asp [D]	1	71
				Missense	G	Asp [D]	1	71
				contig reference	T	Tyr [Y]	1	71
				contig reference	T	Tyr [Y]	1	71
25227335	379	rs730880469	Likely pathogenic		-		1	63
25227341	375	rs17851045	Pathogenic	Missense	C	His [H]	3	61
				Missense	T	His [H]	3	61
				contig reference	A	Gln [Q]	3	61
25227342	374	rs121913240	Pathogenic	Missense	C	Pro [P]	2	61
				Missense	G	Arg [R]	2	61
				Missense	T	Leu [L]	2	61
				contig reference	A	Gln [Q]	2	61
25227343	373	rs121913238	Pathogenic	Missense	A	Lys [K]	1	61
				Missense	G	Glu [E]	1	61
				contig reference	C	Gln [Q]	1	61
25227345	371	rs727503108	Pathogenic	Missense	T	Val [V]	2	60
				contig reference	G	Gly [G]	2	60
25227346	370	rs104894359	Pathogenic	Missense	A	Ser [S]	1	60
				Missense	C	Arg [R]	1	60
				contig reference	G	Gly [G]	1	60
25227348	368	rs104886029	untested	Missense	T	Val [V]	2	59
				contig reference	C	Ala [A]	2	59
25227349	367	rs121913528	Likely pathogenic	Missense	A	Thr [T]	1	59
				Missense	A	Thr [T]	1	59
				Missense	T	Ser [S]	1	59
				Missense	T	Ser [S]	1	59
				contig reference	G	Ala [A]	1	59
				contig reference	G	Ala [A]	1	59
25227351	365	rs104894364	Pathogenic	Missense	T	Ile [I]	2	58
				contig reference	C	Thr [T]	2	58
25227376	340	rs730880470	Uncertain significance	Missense	T	Ser [S]	1	50
				contig reference	A	Thr [T]	1	50
25227386	330	rs904755552		Missense	G	Met [M]	3	46
				contig reference	T	Ile [I]	3	46
25245277	300	rs727503109	Likely pathogenic	Missense	G	Met [M]	3	36
				contig reference	A	Ile [I]	3	36
25245284	293	rs104894366	Pathogenic	Missense	G	Arg [R]	2	34
				Missense	T	Leu [L]	2	34
				contig reference	C	Pro [P]	2	34
25245309	268	rs794727277	Uncertain significance	Missense	T	Tyr [Y]	1	26
				contig reference	A	Asn [N]	1	26
252453	264	rs7547674		frame	T	Ser	3	25

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
12		87		shift		[S]		
				contig reference	-	Gln [Q]	3	25
25245317	260	rs730880472	Likely pathogenic	Missense	G	Arg [R]	2	23
				contig reference	T	Leu [L]	2	23
25245320	257	rs727503110	Pathogenic	Missense	G	Arg [R]	2	22
				Missense	T	Leu [L]	2	22
				contig reference	A	Gln [Q]	2	22
25245321	256	rs121913236		Missense	A	Lys [K]	1	22
				contig reference	C	Gln [Q]	1	22
25245328	249	rs121913538	Uncertain significance	Missense	C	Phe [F]	3	19
				Missense	T	Phe [F]	3	19
				contig reference	G	Leu [L]	3	19
25245345	232	rs104894365	Pathogenic	Missense	A	Ile [I]	1	14
				contig reference	G	Val [V]	1	14
25245347	230	rs112445441	Pathogenic	Missense	A	Asp [D]	2	13
				contig reference	G	Gly [G]	2	13
25245348	229	rs121913535	Pathogenic	Missense	A	Ser [S]	1	13
				Missense	C	Arg [R]	1	13
				Missense	T	Cys [C]	1	13
				contig reference	G	Gly [G]	1	13
25245350	227	rs121913529	Pathogenic	Missense	A	Asp [D]	2	12
				contig reference	G	Gly [G]	2	12
25245351	226	rs121913530	Pathogenic	Missense	A	Ser [S]	1	12
				Missense	C	Arg [R]	1	12
				Missense	T	Cys [C]	1	12
				contig reference	G	Gly [G]	1	12
25245355	221	rs606231202	Pathogenic		TGG	Gly [G]	2	10
25245370	207	rs104894361	Pathogenic	Missense	T	Asn [N]	3	5
				contig reference	A	Lys [K]	3	5
25245372	205	rs193929331	Pathogenic	Missense	G	Glu [E]	1	5
				contig reference	A	Lys [K]	1	5

While the second sequence extended from position 48016 to 48323 showed 83 missense mutations with 20 pathogenic once, 4 frame shift mutations, 4 insertion mutations, and no deletion mutation was observed. More elaboration is given in table (2).

Table 2: Types and locations of mutations identified at position 48016 - 48323 of chromosome 12, KRAS gene.

Chr. position	**mRNA pos**	**dbSNP rs# cluster id**	**Clinical Significance**	**Function**	**dbSNP allele**	**Protein residue**	**Codon pos**	**Amino acid pos**
25209808	746	rs775000854		missense	A	Tyr [Y]	2	185
				contig reference	G	Cys [C]	2	185
25209822	730	rs749587181			-		1	180
25209822	732	rs762532538		missense	C	Asn [N]	3	180
				contig reference	G	Lys [K]	3	180
25209826	728	rs763736188		missense	G	Arg [R]	2	179
				contig reference	A	Lys [K]	2	179
25209828	725	rs587782954	Uncertain significance		GAA	Lys [K]	2	180
25209829	723	rs397517043	Uncertain significance		-		3	180
25209842	712	rs900508259		missense	A	Ser [S]	1	174
				contig reference	G	Gly [G]	1	174
25209854	700	rs369501492	other	missense	T	Leu [L]	1	170
				contig reference	A	Met [M]	1	170
25209871	683	rs372793780	Uncertain significance	missense	A	Gln [Q]	2	164
				contig reference	G	Arg [R]	2	164
25209884	670	rs757674707		missense	A	Ile [I]	1	160

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
				contig reference	G	Val [V]	1	160
25209892	662	rs794727720	Uncertain significance	missense	G	Cys [C]	2	157
				contig reference	A	Tyr [Y]	2	157
25209894	660	rs104894362	Pathogenic	missense	G	Leu [L]	3	156
				contig reference	C	Phe [F]	3	156
25209896	658	rs397517042	Pathogenic	missense	A	Ile [I]	1	156
				missense	G	Val [V]	1	156
				contig reference	T	Phe [F]	1	156
25209898	656	rs755177746		missense	G	Gly [G]	2	155
				contig reference	C	Ala [A]	2	155
25209904	650	rs104894360	Pathogenic	missense	G	Gly [G]	2	153
				missense	T	Val [V]	2	153
				contig reference	A	Asp [D]	2	153
25209907	647	rs104894367	Pathogenic	missense	G	Gly [G]	2	152
				contig reference	T	Val [V]	2	152
25209908	646	rs397517041	Likely pathogenic	missense	T	Phe [F]	1	152
				contig reference	G	Val [V]	1	152
25225625	631	rs387907206	Pathogenic	missense	G	Glu [E]	1	147
				contig reference	A	Lys [K]	1	147
25225628	628	rs121913527	Uncertain significance	missense	A	Thr [T]	1	146
				contig reference	G	Ala [A]	1	146
25225641	615	rs138669124		missense	A	Leu [L]	3	141
				contig reference	T	Phe [F]	3	141
25225651	605	rs754870563		missense	A	Glu [E]	2	138
				contig	G	Gly	2	138

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
				reference		[G]		
25225652	604	rs778702415		missense	A	Arg [R]	1	138
				contig reference	G	Gly [G]	1	138
25225657	599	rs757816355		missense	A	Asn [N]	2	136
				contig reference	G	Ser [S]	2	136
25225663	593	rs373500216		missense	G	Gly [G]	2	134
				contig reference	C	Ala [A]	2	134
25225675	581	rs730880473	Uncertain significance	missense	T	Val [V]	2	130
				contig reference	C	Ala [A]	2	130
25225681	575	rs746609817		missense	G	Arg [R]	2	128
				contig reference	A	Lys [K]	2	128
25225684	572	rs781634879		missense	G	Arg [R]	2	127
				contig reference	C	Thr [T]	2	127
25225694	562	rs575569675		missense	T	Ser [S]	1	124
				contig reference	A	Thr [T]	1	124
25225709	547	rs730880471	Likely pathogenic	missense	A	Asn [N]	1	119
				contig reference	G	Asp [D]	1	119
25225713	543	rs770248150		missense	C	Asn [N]	3	117
				contig reference	A	Lys [K]	3	117
25225717	539	rs202247812	untested	missense	G	Ser [S]	2	116
				contig reference	A	Asn [N]	2	116
25225730	526	rs775836436		missense	A	Ile [I]	1	112
				contig reference	G	Val [V]	1	112
25225742	514	rs763553461		missense	T	Tyr [Y]	1	108
				contig refer-	G	Asp [D]	1	108

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
				ence				
25227234	482	rs727503106	Likely pathogenic	missense	A	Lys [K]	2	97
				contig reference	G	Arg [R]	2	97
25227262	454	rs953088090		missense	G	Glu [E]	1	88
				contig reference	A	Lys [K]	1	88
25227263	453	rs397517038	Uncertain significance	frame shift	-	Asn [N]	3	88
				contig reference	T	Lys [K]	3	88
25227288	428	rs868857258		missense	C	Pro [P]	2	79
				contig reference	T	Leu [L]	2	79
25227294	422	rs756890312		missense	C	Ala [A]	2	77
				contig reference	G	Gly [G]	2	77
25227300	416	rs780974222		missense	C	Ala [A]	2	75
				contig reference	G	Gly [G]	2	75
25227304	412	rs770020203		missense	G	Ala [A]	1	74
				contig reference	A	Thr [T]	1	74
25227308	408	rs104886028	untested	missense	A	Ile [I]	3	72
				contig reference	G	Met [M]	3	72
25227310	406	rs727504662	Pathogenic	missense	T	Leu [L]	1	72
				contig reference	A	Met [M]	1	72
25227313	403	rs387907205	Likely pathogenic	missense	C	His [H]	1	71
				missense	C	His [H]	1	71
				missense	G	Asp [D]	1	71
				missense	G	Asp [D]	1	71
				contig reference	T	Tyr [Y]	1	71
				contig reference	T	Tyr [Y]	1	71

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
25227335	379	rs730880469	Likely pathogenic		-		1	63
25227341	375	rs17851045	Pathogenic	missense	C	His [H]	3	61
				missense	T	His [H]	3	61
				contig reference	A	Gln [Q]	3	61
25227342	374	rs121913240	Pathogenic	missense	C	Pro [P]	2	61
				missense	G	Arg [R]	2	61
				missense	T	Leu [L]	2	61
				contig reference	A	Gln [Q]	2	61
25227343	373	rs121913238	Pathogenic	missense	A	Lys [K]	1	61
				missense	G	Glu [E]	1	61
				contig reference	C	Gln [Q]	1	61
25227345	371	rs727503108	Pathogenic	missense	T	Val [V]	2	60
				contig reference	G	Gly [G]	2	60
25227346	370	rs104894359	Pathogenic	missense	A	Ser [S]	1	60
				missense	C	Arg [R]	1	60
				contig reference	G	Gly [G]	1	60
25227348	368	rs104886029	untested	missense	T	Val [V]	2	59
				contig reference	C	Ala [A]	2	59
25227349	367	rs121913528	Likely pathogenic	missense	A	Thr [T]	1	59
				missense	A	Thr [T]	1	59
				missense	T	Ser [S]	1	59
				missense	T	Ser [S]	1	59
				contig reference	G	Ala [A]	1	59
				contig reference	G	Ala [A]	1	59
25227351	365	rs104894364	Pathogenic	missense	T	Ile [I]	2	58
				contig reference	C	Thr [T]	2	58

Chr. position	mRNA pos	dbSNP rs# cluster id	Clinical Significance	Function	dbSNP allele	Protein residue	Codon pos	Amino acid pos
25227376	340	rs730880470	Uncertain significance	missense	T	Ser [S]	1	50
				contig reference	A	Thr [T]	1	50
25227386	330	rs904755552		missense	G	Met [M]	3	46
				contig reference	T	Ile [I]	3	46
25245277	300	rs727503109	Likely pathogenic	missense	G	Met [M]	3	36
				contig reference	A	Ile [I]	3	36
25245284	293	rs104894366	Pathogenic	missense	G	Arg [R]	2	34
				missense	T	Leu [L]	2	34
				contig reference	C	Pro [P]	2	34
25245309	268	rs794727277	Uncertain significance	missense	T	Tyr [Y]	1	26
				contig reference	A	Asn [N]	1	26
25245312	264	rs754767487		frame shift	T	Ser [S]	3	25
				contig reference	-	Gln [Q]	3	25
25245317	260	rs730880472	Likely pathogenic	missense	G	Arg [R]	2	23
				contig reference	T	Leu [L]	2	23
25245320	257	rs727503110	Pathogenic	missense	G	Arg [R]	2	22
				missense	T	Leu [L]	2	22
				contig reference	A	Gln [Q]	2	22
25245321	256	rs121913236		missense	A	Lys [K]	1	22
				contig reference	C	Gln [Q]	1	22
25245328	249	rs121913538	Uncertain significance	missense	C	Phe [F]	3	19
				missense	T	Phe [F]	3	19
				contig reference	G	Leu [L]	3	19
25245345	232	rs104894365	Pathogenic	missense	A	Ile [I]	1	14
				contig reference	G	Val [V]	1	14
25245347	230	rs112445441	Pathogenic	missense	A	Asp [D]	2	13
				contig reference	G	Gly [G]	2	13
25245348	229	rs121913535	Pathogenic	missense	A	Ser [S]	1	13
				missense	C	Arg [R]	1	13
				missense	T	Cys [C]	1	13
				contig reference	G	Gly [G]	1	13
25245350	227	rs121913529	Pathogenic	missense	A	Asp [D]	2	12
				contig reference	G	Gly [G]	2	12
25245351	226	rs121913530	Pathogenic	missense	A	Ser [S]	1	12
				missense	C	Arg [R]	1	12
				missense	T	Cys [C]	1	12
				contig reference	G	Gly [G]	1	12
25245355	221	rs606231202	Pathogenic		TGG	Gly [G]	2	10
25245370	207	rs104894361	Pathogenic	missense	T	Asn [N]	3	5
				contig reference	A	Lys [K]	3	5
25245372	205	rs193929331	Pathogenic	missense	G	Glu [E]	1	5
				contig reference	A	Lys [K]	1	5

Although *KRAS* activation mutation has been shown to be associated with increased proliferation in cancer cell lines, its exact effect in human cancer is relatively unclear. In a few studies, *KRAS* mutation has been shown to be associated with a diffuse proliferation pattern, polypoid growth, and high cytologic grade in colorectal adenomas and early cancers. In a small study, a KRAS mutation was suggested to be associated with decreased apoptosis Suehiro *et al.*, 2008.

However, most of the mutations detected were of missense type where the protein configuration was altered in several ways, non-significant type that is related to the redundancy of the genetic code, uncertain type where the effect of the mutation is nor fully studied, benign type where there is no effect on the product protein, likely pathogenic ad likely benign which may an indicator pre – cancer initiation, uncertain that is not studied yet, and pathogenic which resulted in cancer initiation and proliferation.

Frame shift mutation, insertion and deletion were detected in low rate in both sequences. Even with such low frequency, it still associated with pathogenic effect on KRAS gene.

Association of gender and age with colorectal cancer: In most cases, CRC was found in elderly people, mostly detected in men more than women. Table (3) and table (4) show the percentage of CRC according to the age and gender respectively.

Table 3: Manifestation of CRC according to the age group.

Age group (years)	Number of patients	Percentage (%)
40 — 50	35	18.00
51 — 60	65	32.50
61 — 70	100	50.00
Total	200	100.0

Table 4: Distribution of CRC according to the gender

Gender	No. of patients with CRC
Male	130
Women	70

Considering potential gender differences in recommended age ranges for CRC screening, non-epidemiological criteria, such as complexity of guidelines, also have to be taken into account (Lieberman, 2005). One might argue that gender-specific recommendations might add another layer of complexity, which could be a barrier against use of CRC screenings. However, from the patients' point of view, schedules for cancer screening are gender specific anyway; given that some of the most widely used screening measures refer to female (breast, cervical) and male (prostate) cancers.

Discussion: KRAS mutation is a critical point in initiation of cancer in different parts of the colon. The lost of cell cycle control may result in abnormal growth of tumor lumps with undifferentiated cell which represent cancer in this organ.

Most literatures mentioned mutations at codon 12 and 13 (Brenner *et al.*, 2007, Gado *et al.*, 2014) which was detected at Arabian population in Egypt, UAE, and Saudi Arabia, while in Iraqi population we focused in previous study (Rehab *et al.*, 2018) on detection other types of mutations and their accumulation at non affected site which was mainly blood on which we were able to amplify most of parts at exon 2 10 specifically designed primers, while in this study; only 2 primers were able to amplify specific parts on exon2 and the other primers failed to that suggesting significant rate of genetic change in this exon associated with CRC.

However such change was diagramed in figure (1) and figure (2) which show the distribution of pathogeneic mutation at the site under study.

Fig. 1: Distribution of lethal mutation in KRAS gene depending the sequence obtained from primer PK1.

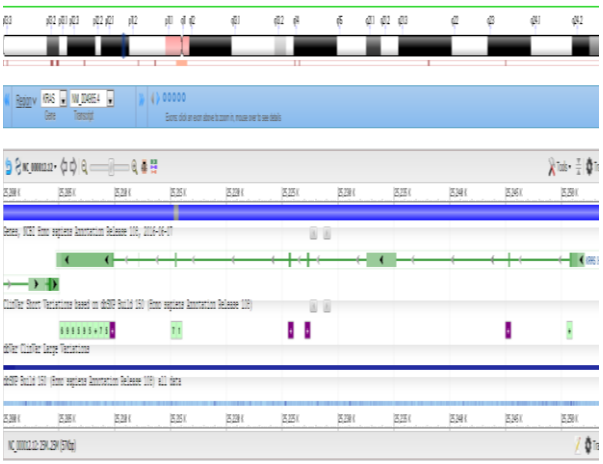

Fig. 2: Distribution of lethal mutation in KRAS gene depending the sequence obtained from primer PK2.

Detailed molecular analysis of both obtained sequences showed significant change in ORFs. Wild type sequence related to PK1 primer showed no ORFs while that obtained with the same primer from tumor tissue showed the development of two ORFs at specific locations given in table (4).

Table (5). Detailed analysis of ORFs detected in the sequence obtained from primer PK1

Table 5: Detailed analysis of ORFs detected in the sequence obtained from primer PK1.

Label	Strand	Frame	Stop	Length (nt | aa)
ORF2	-	**3**	**73**	**135 | 44**
ORF1	+	1	252	81 | 26

The same detailed analysis was conducted to the sequence obtained from primer PK2 which showed the presence of 21 ORFs elaborated in table (6).

Table 6: Detailed analysis of ORFs detected in the sequence obtained from primer PK2.

Label	Strand	Frame	Start	Stop		Length (nt | aa)
ORF5	**+**	**3**	**1440**	**1706**		**267 | 88**
ORF7	+	3	3249	3410		162 | 53
ORF3	+	1	3283	3441		159 | 52
ORF12	-	2	3609	3472		138 | 45
ORF2	+	1	1459	1593		135 | 44
ORF14	-	2	282	148		135 | 44
ORF16	-	3	3380	3255		126 | 41
ORF10	-	1	1726	1604		123 | 40
ORF17	-	3	3119	3018		102 | 33
ORF15	-	3	3569	3468		102 | 33
ORF21	-	3	803	705		99 | 32
ORF9	-	1	2110	2021		90 | 29
ORF20	-	3	1115	1026		90 | 29
ORF18	-	3	1748	1662		87 | 28
ORF4	+	1	3499	3582		84 | 27

ORF8	-	1	2830	2750		81 | 26
ORF6	+	3	2640	2720		81 | 26
ORF19	-	3	1532	1452		81 | 26
ORF1	+	1	247	327		81 | 26
ORF11	-	1	1411	1334		78 | 25
ORF13	-	2	681	604		78 | 25

Such dramatic change in finding new ORFs among the gene sequence indicates the production of new types of proteins that are not found in the wild type, which may lead in progressive proliferation in cancer cells without control as a result of stopping control signal in these cells. Age and diet are considered as risk factors in CRC, A longitudinal study from Europe, the EPIC, demonstrated an increased risk for people who consumed more than 160g of red or processed meat per day. Norat *et al.*, 2005. In another prospective study from the United States, the HPFS, there was an increased risk of approximately threefold for those who consumed more than five servings per week of red meat. The comparison group ate less than one serving per month. In a study that combined the NHS and the HPFS, red meat was a risk for colon but not rectal cancer Wei *et al.*, 2004.

In the CPS II Nutrition Cohort, Chao *et al.* 2005observed an increased risk of red meat for distal and rectal CRC. Such diet and food with high spice, fat, and salt is a criteria of male food which may increase the risk of CRC in male.

Ethanol-based beverages have been thought to increase the risk of rectal and colon cancer through a variety of mechanisms including abnormal DNA methylation and repair, induce cytochrome p450 enzymes to increase carcinogen production and alter bile acid composition Thygesen *et al.*, 2008. An analysis from the HPFS showed that there was a positive correlation between risk of CRC and alcohol in men. This risk increased after 15 g per day which is about one drink per day. In a study that combined the NHS and HPFS, alcohol increased the risk of colon but not rectal cancer. Data from the EPIC trial demonstrated that after controlling for smoking and other known risk factors, alcohol increased the risk of CRC Ferrari *et al.*, 2007. However in a sub population of the EPIC study, Park *et al.* observed no risk association between alcohol and CRC. They did find a decrease in risk associated with wine. A study, which combined eight studies for a total of a half a million patients, observed an increased risk for patients who had more than two alcohol beverages per day. In that study, all forms of alcohol increased risk including wine. However, overall it appears that regular alcohol consumption may be associated with an increased risk for CRC and that moderation of alcohol beverage intake may be the best strategy Park *et al.*, 2009. Although most studies have observed an increased risk for men with regard to advanced colorectal neoplasia as well as CRC, the overall lifetime risk for CRC for men and women is numerically similar Roy *et al.*, 2009. In addition, women have a 5-year lag with respect to incidence of CRC. For example, a woman at 55 has a similar risk to a man at 50 years of age Lieberman *et al.* 2005. With regard to the risk for CRC, Nguyen *et al.* in a meta-analysis observed a twofold increase risk for CRC and advanced adenomas in men as compared to women Nguyen *et al.*, 2009. Furthermore, in the concern trial, Phipps *et al.* 2013 observed a lower risk for advanced adenomas in women compared to men. A study by Bressler *et al.* 2009 showed that women were more likely than men to have subsequent CRC after having a colonoscopy. Thus it appears that changes with respect to how we screen women as compared to men may be reasonable. More data, however, is needed to explain the paradox of different advanced adenoma rates but similar CRC rates for the genders.

Ethics approval and consent to participate:
This study did not include any human subjects and did not reveal any personal information regarding patients from which samples were collected.

Consent for publication: This work did not include any personal, written information, pictures and videos to any person.

Availability of data and material: All data and materials used in this study are available and stored at College of Biotechnology, Al-Nahrain University, and Biotechnology Research center, Al-Nahrain University.

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4. Conclusion

Colorectal cancer posed a great threat in Iraqi population and it is highly manifested among men rather than women. Presence of specific pathogenic mutations altred KRAS gene expression causing defected function of this gene. This may further developed to construct a bio-marker for early detection of CRC.

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