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Research Article

MUTATION ANALYSIS OF *K RAS* GENE IN COLORECTAL CANCER IN IRAQI PATIENTS

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ABSTRACT

The high frequency of *Kras* mutations and the observation that they mostly appear during early stages of tumor progression provide strong argument supporting a causative role of *Kras* in human tumorigenesis. A fragment of *Kras* gene with molecular size 108bps, amplified by conventional PCR was detected in extracted of blood genomic DNA from (29) patients with colorectal cancer (CRC) collected from teaching hospitals of some Iraqi governorates. The patients average aged was 46.86 years, male to female ratio was 65.5% to 34.5% respectively. The sample of the patients were compared with 4 healthy as a control which were processed for detection of mutations and confirmed by PCR-Sequences. The sequences analysis of the variant regions for the amplification of the defect gene were 20.68% (6 out of 29) of the patients as compared with the Sequences of Gene Bank.

Keywords: Colorectal cancer, mutated *K ras* gene, sequencing *K ras* gene.

INTRODUCTION

GTPase *K ras* also termed *KRAS* also known as V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog and *Kras*, is a protein that in humans is encoded by the *Kras* gene [1, 2]. The protein product of the normal *Kras* gene performs an essential function in normal tissue signaling, and the mutation of a *Kras* gene is an essential step in the development of many cancers [3]. This proto-oncogene is a Kirsten ras oncogene homolog from the mammalian ras gene family. A single amino acid substitution, and in particular a single nucleotide substitution, is responsible for an activating mutation. The transforming protein that results is implicated in various malignancies, including lung adenocarcinoma, mucinous adenoma, ductal carcinoma of the pancreas and colorectal carcinoma. Several germline *Kras* mutations have been found to be associated with Noonan syndrome and cardio-facio-cutaneous syndrome [4,5]. The *Kras* protein is activated transiently as a response to extracellular signals such as growth factors, cytokines and hormones that stimulate cell surface receptors [6]. Somatic *Kras* mutations are found at high rates in leukemias, colon cancer [7 ,8] pancreatic cancer and lung cancer [9]. The Fearon and Vogelstein model assumes the involvement of the *APC* gene in adenoma formation and the *Kras* oncogene in the transition from intermediate adenomas to carcinomas in sporadic CRC [10]. Also studies were suggested, that a mutated *Kras* gene contributes to the transition of an intermediate adenoma to a late adenoma or carcinoma [11]. The properties of Ras function is a switch between an inactive state, in which the proteins are bound to guanosine-diphosphates (GDP) and an active state in which conversion to guanosine-triphosphates (GTP) has occurred. This transit is controlled by two types of regulatory proteins: GDP–GTP exchange factors that catalyse the GDP–GTP exchange and GTPase-activating proteins that enhance the intrinsic capacity of Ras proteins to hydrolyse GTP into GDP, thereby returning Ras to the inactive state [12].

MATERIAL & METHODS

Samples and DNA extraction

Blood samples were obtained from (29) of Iraqi patients suffered from colorectal cancer average aged 46.86 years' male to female ratio was 65.5% to 34.5% respectively, compared with 4 healthy as a control. Samples from 20 healthy individuals were used as a control group. In a total of 4 ml whole blood was collected into an Ethylene di amine tetra acetic acid (EDTA) tube. The samples were stored at (-20°C) until further processing. The DNA was extracted by blood DNA extraction kit using the Geneaid (Koria) as mentioned by leaflet kit according to the manufacturer's protocol. Experiments on human subjects are approved by an Institutional Ethical Committee.

Amplification of exon1 of *K ras* gene

The amplification conditions for the *K ras* gene were optimized by the conventional PCR assay by using (Applied-PCR 9700; BioSystem/USA) company with specific primers sequences for the gene. A fragment of *K ras* gene with molecular size (108) bps for exon1 was amplified using a forward primer (5-GACTGAATATAAACTTGTGG-3) and a reverse primer (5-CTATTGTTGGATCATATTCG-3) [13]. Template of DNA and primers were thawed before use in moderate cold conditions. And the two above mentioned were added into the *AccuPower* ® *Profi Taq* PCR Premix tubes (BioneerAccuPower/Korea). A condition work of lyophilized PCR premix 50 µl (as leaflet kit). Template DNA 1 µl, Forward primer 1 µl, Reverse primer PCR 1 µl deionised distilled water was completed into the AccuPower® Profi Taq PCR Premix tubes to total volume of 50 µl and lyophilized blue pellet was completely dissolved and spin down by vortex. The thermal cycling conditions were as follows: Denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 secs, 53°C for 30 secs and 72°C for 30 secs with

final incubation at 72°C for 10 min The PCR product were separated by1.2% agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet light (302 nm).

Sequencing and sequence alignment

Sequencing of exon1of *K ras* gene was performed for all the samples by a source of a company NICEM/USA (machine type AB 13730XL) of Applied Biosystems Macro. Homology search was conducted using Basic local alignment search tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online and BioEdit program [14]. The results were compared with data obtained from Gene Bank published for DNA translation from Expasy program [15].

Statistical analysis

The statistical analysis is a very important final step in the research to analyses and evaluates the obtained results. Medical statistics of this study was conducted via computer based statistical program which was: X2 for Windows computer package. The statistical analysis tests used in this were as follows: P value < 0.01 is considered a significant correlation.

RESULT AND DISCUTION

The high frequency of *K ras* mutations and the observation that they mostly appear during early stages of tumor progression provide strong argument supporting a causative role of *Kras* in human tumorigenesis [16]. *K ras* point mutation with molecular size 108 bps which showed in figure (1) amplified by a specific PCR primers for exon1 mentioned above was detected in extracted of blood genomic DNA of (29) patients with CRC average aged 46.86 years (males to females ratio was 65.5% to 34.5%) respectively, compared with 4 healthy as a control. The result of our study in agreement with the study of Shine and colleagues, (1996).

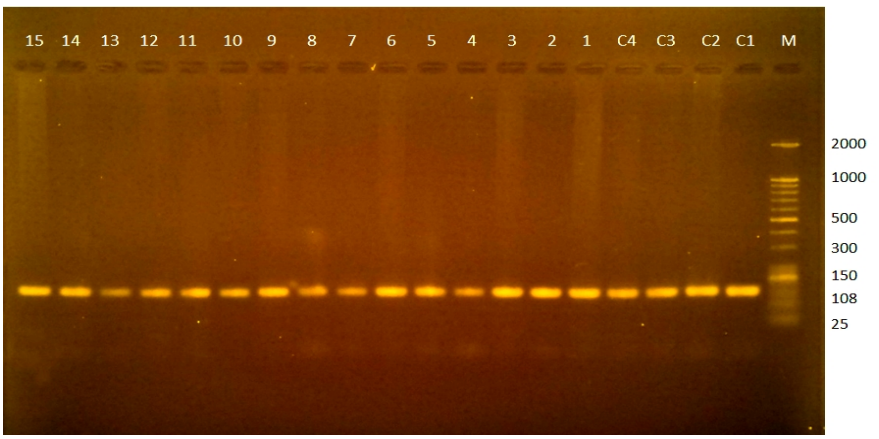

Figure 1: Gel electrophoresis of amplified *K ras* gene, exone1 of CRC patients (1-15) and healthy (C1-C4). Bands were fractionated by electrophoresis on a 1.2 % agarose gel (1.5hr., 5V/cm, 0.5X TBE buffer) and visualized under U.V. light after staining with ethidium bromide dye. Lane M (25bp/Bioneer ladder).

Sequencing of *Kras* gene was performed to detect variant defection which related to development of colorectal cancer for these patients through the DNA products. Sequences alignment using BLAST and BioEdit showed the 100% similarity or homology of healthy sample with wild type of the *K ras* gene of *H. sapiens* from the Gene Bank figure (2).

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (KRAS), RefSeqGene on chromosome 12 Sequence ID: ref|NG_007524.1|Length: 52675Number of Matches: 1Related InformationMap Viewer-aligned genomic contextRange 1: 10568 to 10646GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
147 bits(79)	2e-32	79/79(100%)	0/79(0%)	Plus/Plus

```
Query 1      CTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 10568  CTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACG  10627

Query 61     AATATGATCCAACAATAGA  79
             ||||||||||||||||||
Sbjct 10628  AATATGATCCAACAATAGA  10646
```

Figure 2: Sequence of sense flanking the partial *K RAS* gene, exon 1 for healthy as compared with standard *K-ras* (*KRAS*) gene, exon 1 obtained from Gene Bank.

The sequence analysis of the variant regions for the amplification of the defect gene was 20.68% (6 out of 29) for patients as compared with a Sequence of gene bank. The *K ras* gene from (29) of patients with CRC showed variant compatibility with the wild type sequences of *K ras* gene from Gene Bank as shown in Figure (3) of A-D).

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (KRAS), transcript variant X1, mRNA Sequence ID: ref|XM_006719069.1|Length: 812Number of Matches: 1Related InformationGene-associated gene detailsMap Viewer-aligned genomic contextRange 1: 175 to 248GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
126 bits(68)	3e-26	72/74(97%)	0/74(0%)	Plus/Minus

```
Query 7    TTCTGAAATAGCTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGGTCCAACTACCACA  66
           ||||||| ||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct 248  TTCTGAATTAGCTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGCTCCAACTACCACA  189

Query 67   AGTTTATATTCAGT  80
           ||||||||||||||
Sbjct 188  AGTTTATATTCAGT  175
```

Figure 3.A: Sequences of the partial *K RAS* gene, exon 1 for patients revealed a transversion Cytocine to Guanocine (C>G), changes nucleotide GCT>GGT frome Alaninen>Glycine, Substitution nucleotide at location 176 obtained from Gene Bank.

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (KRAS), RefSeqGene on chromosome 12 Sequence ID: ref|NG_007524.1|Length: 52675Number of Matches: 1Related Information Map Viewer-aligned genomic contextRange 1: 10568 to 10646GenBank Graphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
128 bits(69)	7e-27	76/79(96%)	2/79(2%)	Plus/Plus

```
Query 3      CTGGCGGCGTA-GC-AGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACG  60
             |||| ||||| || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct 10568  CTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACG  10627

Query 61     AATATGATCCAACAATAGA  79
             ||||||||||||||||||
Sbjct 10628  AATATGATCCAACAATAGA  10646
```

Figure 3.B: Sequences of the partial *K RAS* gene, exon 1 for patients revealed 1- Deletio Guanocine (TAG> TA-) lead to stop codon, location at 10579. 2-Deletion of adenine (A>-) lead to change Lycine > Glutamine, GCA>CAG at location 10582 obtained from Gene Bank.

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (KRAS), RefSeqGene on chromosome 12 Sequence ID: ref|NG_007524.1|Length: 52675Number of Matches: 1Related InformationMap Viewer-aligned genomic contextRange 1: 10579 to 10646GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
119 bits(64)	4e-24	67/68(99%)	1/68(1%)	Plus/Plus

```
Query 10     GGC-AGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCA  68
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 10579  GGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCA  10638

Query 69     ACAATAGA  76
             ||||||||
Sbjct 10639  ACAATAGA  10646
```

Figure 3.C: Sequences of the partial *K RAS* gene, exon 1 for patients revealed deletion of adenine (A>-) through change of nucleotide (AAG>AGA) frome Lycine>Arginine at location 10582 obtained from Gene Bank.

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (KRAS), RefSeqGene on chromosome 12 Sequence ID: ref|NG_007524.1|Length: 52675Number of Matches: 1Related Information Map Viewer-aligned genomic contextRange 1: 10573 to 10645GenBankGraphics Next Match Previous Match

Alignment statistics for match #1

Score	Expect	Identities	Gaps	Strand
122 bits(66)	3e-25	71/73(97%)	2/73(2%)	Plus/Plus

```
Query 5      GGCGTA-GC-AGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATAT  62
             |||||| || ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 10573  GGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATAT  10632

Query 63     GATCCAACAATAG  75
             |||||||||||||
Sbjct 10633  GATCCAACAATAG  10645
```

Figure 3.D: Sequences of the partial *K RAS* gene, exon 1 for patients revealed: 1-Deletion guanocine (G>-) changes of nucleotide GGC>GCA from Gly>Ala at location 10579 2-Deletion adenine (A>-) changes of nucleotide AAG>GAG from Lyc>Glu at location 10582 obtained from Gene Bank.

The results of a mutated of *K ras* gene, revealed through the figure (3: A, B) which showed an increased of the frame shift mutation explained by guanocine deletion (G>-) lead to stop codon so as with change GGC>GCA and deletion of adenine (A>-) with substitution of the specially bases A>G through the types of the nucleotides of patients at nucleotide location (10582) as shown in table (1) which explain 6 types of mutations mostly frame shift (deletion) as elucidated in figure (3: B- D). The frame shift mutation (deletion) has more frequency in exon1 of *K ras*, and these results which supported by the most frequently observed types of mutations by A>G transitions or G>C transversions [17,18].

Table 1: Types of mutations, location of nucleotides and amino acid changes in *K ras* gene, exon 1 in patients with CRC

No	Location of nucleotides change	Nucleotide change	No. Of sampe	amino acid change	Predicted effect	Type of mutation
1	C > G 176	GCT>GGT	1(16.67)	Ala>Gly	Missense	Transversion
2	G>- 10579 A>- 10582	TAG>TA- GCA>CAG	3(50.00)	Stop codon Lyc > Glu	Frame shift	Deletion
3	G>- 10579 A>- 10582	GGC>GCA AAG>GAG	1(16.67)	Gly>Ala Lyc>Glu	Frame shift	Deletion
4	A>- 10582	AAG>AGA	1(16.67)	Lyc > Arg	Frame shift	Deletion
	Ch-square-χ^2 ** (P≤0.01)	---	10.409 **	---	---	---

The previous studies about *K ras* gene in Iraqi patients enrolled exon 2 and denoted the highly percent of transition mutation in guanocine with adenine base, which the tandem Glycine 12-Glycine 13 (G12-G13) accounts for about 99% of the mutations detected (86% and 13%) respectively, whereas mutations affecting the other main hot spot in *K ras* proteins like G>C, G>T, A>T account for the remaining 1% in exon 2, as mentioned by [19, 20].

CONCLUSION

From the above findings about *K ras* gene denoted types of mutations in which affecting for possible colorectal cancer

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