



# International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531  
(Print & Online)

<http://gssrr.org/index.php?journal=JournalOfBasicAndApplied>



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## Detection of Five Novel Mutations in *K-ras* Gene for Iraqi Patients with Bladder Cancer

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### Abstract

The present study was carried out in Genetic Engineering and biotechnology Institute –Baghdad University during a period from November 2014 to November 2015, for detecting the role of genetic alterations of *K-ras* gene in Iraqi patients with bladder cancer. 40 patients with bladder cancer who admitted to Ghazi Al Hariri and the National Al Amal hospitals in Baghdad and 25 apparently healthy persons (age between 20 to 87 years) were included in this study. Total genomic DNA was isolated from blood samples for molecular analysis using specific primers for codons 8 and 61 of the gene *K-ras*. The PCR amplified regions of the *K-ras* codons of healthy and patients showed a molecular weight of about 161 bp. The analysis of mutation using restriction fragment length polymorphism (RFLP) was performed on PCR products of *K-ras* codons using *Hpa II* enzyme. These results showed that the PCR amplified regions of the *K-ras* codons have no restriction site for enzyme which occurring in the normal allele also.

The REFLP molecular analysis of *K-ras* codons gene of patient samples revealed that there is no mutation in restriction site of enzyme and the same result showed by healthy samples. The DNA sequencing analysis of the *K-ras* gene PCR products revealed that among 40 patients included in this study, five mutations were detected in codon 8 and 61 included g.28532-33 AT>TG in codon 8 and g.28542 del G, g.28543 A>C, g.28544 A>C in codon 12. The more frequent mutation was g.28542 del G and g.28543 A>C which detected in 15 patients for each one, followed by g.28544 A>C and g.28532-33 AT>TG which detected in 9 patients for each one also. Also the results showed that nine smoker patients were with *K-ras* mutations.

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These results suggest that carcinogens exist in cigarettes could be induce mutations in *K-ras* gene.

**Keywords:** Bladder carcinoma; *K-ras*; RFLP; g.28542 del G; g.28543 A>C; g.28544 A>C; g.28532-33 AT>TG.

## 1. Introduction

Bladder cancer (BC) is one of the most common cancers of the urinary tract and a major problem worldwide. The main known risk factors for bladder cancer include cigarette smoking, exposure to industrially related aromatic amines, and intake of drugs such as phenacetin, chlornaphrazine, and cyclophosphamide. These exposures lead to DNA damage which, if remained unrepaired, may result in unregulated cell growth and even cancer [1].

The bladder is the most common site of cancer in urinary tract [2, 3]. Bladder cancer is nearly three time more common in men than women. There has been a 50 % increase in incidence over the past 40 years. According to the world health organization (WHO) in 2008, over 300,000 people were confronted with the disease and more than 40 % (130,000) of them die because of bladder cancer every year, Indeed, the incidence of bladder cancer is the eleventh in the list of all cancer in the world [4, 5].

In Iraq, bladder cancer is fifth most incident from all cancer types, and they were registered (1163) cases in 2011, the men percentage to women was (3\1). For man bladder cancer take number two with (9.26) % from all cancers types, for women bladder cancer take number ten with (2.72) % from all cancers types [6]. According to the latest WHO data published in 2014, Bladder cancer deaths in Iraq reached 984 or 0.67 % of total deaths.

Many c-oncogenes were identified in bladder cancer to be induced or with inappropriate expression due to mutated forms (due to point mutations, chromosomal translocations, gene amplifications, or by the insertion of stronger promoters [7, 8, 9]. At the molecular level, the *ras* gene family are well involve in bladder cancer. They encode highly similar and conserved proteins with a molecular weight of 21 kDa (p21). The main function of the *ras* protein is to induce activation of downstream kinase cascades that results in continuous mitogenic signaling and transformation of immortalized cells [10:238]. Bladder tumors harbor activating mutations in the *ras* family genes in approximately 7-17 % of samples [11]. *K-ras* gene showed to be play an important role in bladder cancer developing [9, 12]. *K-ras* appears to be the most capable of sustaining cancer programs and this would translate into strong selective pressure for mutations of this isoform [13].

The current research aims to determine the point mutations in the *K-ras* gene in bladder cancer patients and the association between disease, *K-ras* mutation and smoking.

## 2. Materials and Methods

The study consisted of 40 subjects with bladder cancer (Transitional cells carcinoma TCC) and 25 subjects control group. Patient samples were obtained from Ghazi Al Hariri and the National Al Amal hospitals in Baghdad. Patient's age ranged from 20 to 87 years while control subjects ages ranged from 20 to 60 years. Blood samples (3-5 ml) were collected from patients and control in EDTA anticoagulant tubes.

Questionnaire form was filled for each patient including; name, age, gender, family history, employment type other diseases and smoking.

### **2.1 DNA extraction**

Total genomic DNA was isolated from the blood, for molecular studies using genomic DNA purification kits (Bioneer) South Korea.

### **2.2 Polymerase chain reaction (PCR) for codon 61 (161 bp)**

The codon 61 region of *K-ras* gene was amplified by PCR using the primers, F '5-GACTGTGTTTCTCCCTTCT'-3 and R '5-TGGCAAATACACAAAGAAAG'-3 and the conditions were, initial denaturation 4 minutes at 95 °C, followed by 35 cycle each of denaturation 30 seconds at 95 °C, annealing 1 minute at 52 °C, extension 30 seconds at 72 °C and a final extension step at 72 °C for 9 minutes [14:94].

PCR products (3 µl) were then separated on 2 % agarose gel with a ladder (100 bp) and visualized.

### **2.3 Screening for mutations in codon 61 using RFLP analysis**

Screening of the *K-ras* mutations at codon 61 was performed by digestion with restriction endonuclease *Hpa II* (Bioneer, Korea). The PCR product (10 µl) was digested with 5 U of restriction enzyme in 20 µl for 3 hours at 37°C, the product was analyzed by 2 % agarose gel then photographed under ultraviolet light.

### **2.4 PCR products sequencing**

The PCR products of the *K-ras* gene codon 61 region (40 samples) and primers were sent for sequencing by MacroGen Company (U.S.A). The sequences of these samples were compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard *K-ras* gene, using (Bioedit) software.

### **2.5 Statistical analysis**

Data management was done using Statistical Package for Social Sciences (SPSS version 12). SPSS was used for analysis and to perform Pearson Chi-square test for statistical significance (P value). The 95% confidence level and confidence intervals were used.

### **2.6 Ethical consent**

The study was approved by Institute of Genetic Engineering and Biotechnology-University of Baghdad and Ministry of Health in Iraq. All study subjects consented to participation by completing the self-administered questionnaire.

### 3. Results

#### 3.1 Subjects data

The characteristics of the patients are summarized in Table (1). The results indicated a significant correlation between the occurrences of bladder cancer with patient’s ages, smoking and non-smoking.

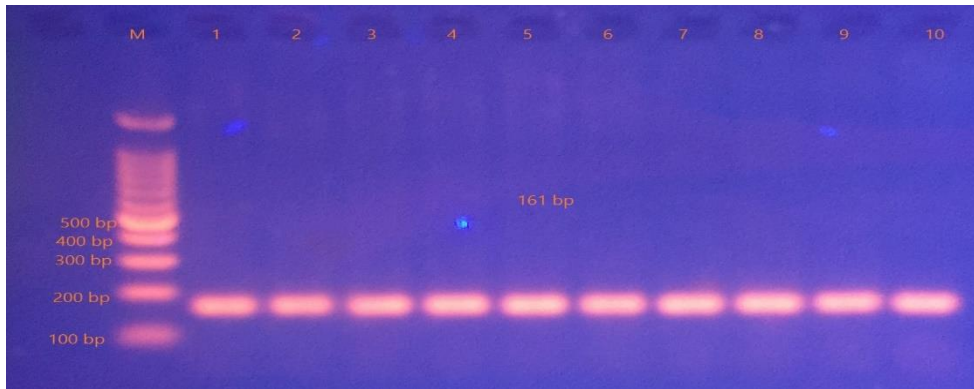
According to data of table 1, there are highly significant difference between numbers of smoker 26 (65.0%) and non-smoker 14 (35.0%) patients within age groups (P<0.01). Group 3 was with high significant ( $X^2$  13.09) (OR1.861), followed by other groups, while there is non-significant difference between smoker and non-smoker at group 1 ( $X^2$  0.00) (OR 0.00).

**Table 1:** profiles of patient samples included in the study according to range of age’s group

Range of ages	Number of patients	Smoker	Non-smoker	Chi-square ( $\chi^2$ )	Odds ratio (OR)
<b>Group 1</b> (21 - 30)	2 (5.0 %)	1 (50.0%)	1 (50.0%)	0.00 NS	0.00
<b>Group 2</b> (31 - 40)	10 (25.0 %)	7 (70.0%)	3 (30.0%)	11.25 **	1.682
<b>Group 3</b> (41 – 50)	8 (20.0 %)	7 (87.5%)	1 (12.5%)	13.09 **	1.861
<b>Group 4</b> (51 – 60)	10 (25.0 %)	4 (40.0%)	6 (60.0%)	7.25 **	1.279
<b>Group 5</b> (over 60)	10 (25.0 %)	7 (70.0%)	3 (30.0%)	11.25 **	1.682
<b>Total and percentages</b>	40 (100.0 %)	26 (65.00 %)	14 (35.00 %)	9.62 **	1.483
<b>Number of patients have mutations from total number</b>	15 (37.5 %)	9 (34.61 %)	6 (42.85 %)		
<b>Chi-square (<math>\chi^2</math>)</b>	8.925 **	12.736 **	11.301**		

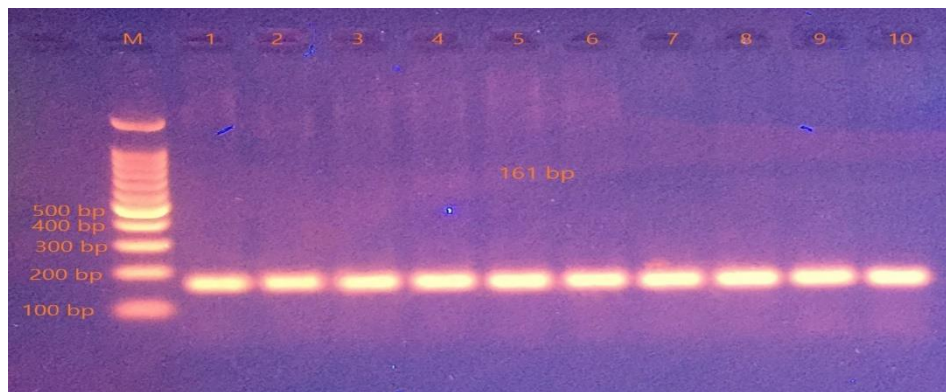
### 3.2 REFLP analysis of *K-ras* codon 61

PCR analysis of *K-ras* codon 61 is shown in Figure 1.



**Figure 1:** Gel electrophoresis of 61 codon of *K-ras* PCR products for healthy and patients with bladder cancer on 2% agarose at 100 volt for 30 minutes stained with Ethidium Bromide and visualized under U.V light. M, ladder, lanes 1-7 patients' bladder cancer samples; lanes 8-10 healthy control samples.

Restriction fragment length polymorphism (RFLP) was performed on PCR products in 61 region of the *K-ras* using *Hpa II* enzymes. There are no restriction sites for *Hpa II* enzyme in the *K-ras* gene sequences but if there were mutations in specific sequences that create new restriction sites then the enzymes will be cut the target sequences in a variable type according to number of new sites. If there were no mutations exist a 161 bp codon 61 PCR product will not be digested by *Hpa II* enzyme as the healthy samples which shown in (Figures 2 and 3).



**Figure 2:** *K-ras* PCR products digested with *Hpa II* enzyme analyzed on a 2% agarose gel at 100volt for 45 minutes stained with Ethidium Bromide and visualized under U.V light. Lane M: Marker, lanes 1 – 7 represent patients with normal pattern, lanes 8 – 10 represent control with normal pattern.

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (KRAS), RefSeqGene on chromosome 12

NCBI Reference Sequence: NG\_007524.1

28487GACTGTGTTTCTCCCTTCTCAGGATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAA  
 CCTGTCTCTTGGATATTCTCGACACAGCAGGTCAAGAGGAGTACAGTGCAATGAGGGACCAG  
 TACATGAGGACTGGGGAGGGCTTTCTTTGTGTATTTGCCA 28647

**Figure 3:** 161 bp PCR *K-ras* product sequence

**3.3 Detection of *K-ras* codon 61 mutations by DNA sequencing**

The bladder cancer patient’s PCR products of the *K-ras* gene codon 61 were screened for mutations by sequencing. The patients sequencing results were compared with human reference *K-ras* gene sequence (http: NCBI Reference Sequence). Among 40 patients included in this study, 15 (37.5 %) patients were with *K-ras* mutations. The mutations detected in *K-ras* 61 region include g.28542 del G, g.28543 A>C, g.28544 A>C, g.28532 A>T and 28533 T>G. The more frequent mutation was 28542 del G which detected in 15 patients (Table 2 and Figures 4 and 5). All mutations in the table are considered novel and not registered in the NCBI. Moreover, the results revealed that six patients were with compound mutations and nine patients were with one mutation (Table 3). Moreover, the results showed that among 26 smoker patients nine (34.6%) were with *K-ras* gene mutations.

**Table 2:** *K-ras* mutations detected in bladder cancer patients.

<i>K-ras/</i> Mutation	Accession No. NG_007524.1 From 28487 to 28647			NCBI Mutation statute
GAA to -AA	g.28542 del G	Framshift	Chang all the amino acids chain	New record
GAA to GCA	g.28543 A>C	Substitution	Glutamic acid to Alanine	New record
GAA to GAC	g.28544 A>C	Substitution	Glutamic acid to Aspartic acid	New record
ATT to TGT	g.28532-33 AT>TG	Substitution	Isoleucine to Cystine	New record

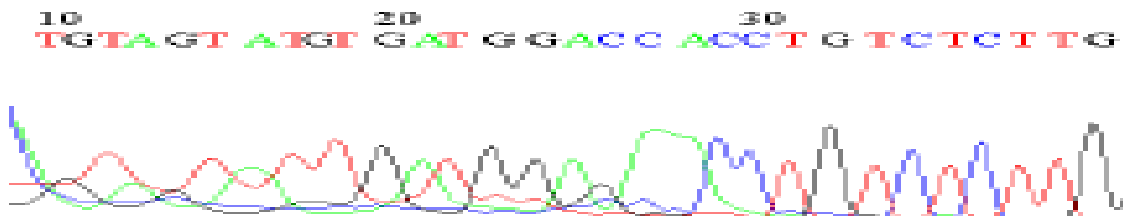
**Table 3:** Compound mutations in bladder cancer patients.

No. of patients / percentage	Gene	Wild type	Mutant type	Site
(3) 20%	<i>K-ras</i>	GAA	-AA	g.28542 del G
		GAA	GCA	g.28543 A> C
(3) 20%	<i>K-ras</i>	ATT	TTT	g.28532 A>T
		ATT	AGT	g,28533 T >G
		GAA	-AA	g.28542 del G
		GAA	GCA	g.28543 A>C
		GAA	GAC	g.28544 A>C

Query 11 GTAGTATGTGATGGA-CCACCTGTCTCTTGGATATTCTCGACACAGCAGGTCAAGAG 66



Sbjct 28527 GTAGTAATTGATGGAGAAACCTGTCTCTTGGATATTCTCGACACAGCAGGTCAAGAG  
28583



**Figure 4:** Sites g.28532 A>T, g.28533 T >G, g.28542 del G, g.28543 A>C and g.28544 A>C, nucleotide sequence (forward) in *K-ras* codon 61

#### 4. Discussion

Bladder cancer (BC) is generally a disease of elderly people and incidence of bladder carcinoma increases with age [2]. Bladder cancer is rare in people younger than 50 years of age, even though it can occur at any age [15]. The current results showed that bladder cancer can effect most ages and distributed from age 30 years to more than 60 years. Such results were also detected in our previous studies on another two groups of Iraqi bladder cancer patients [8, 9]. Young age bladder cancer patients may indicate a kind of exposure to environmental hazardous and/or toxic substances or could be due to cigarettes smoking or novel agents. Such conclusion was also reached by Al-Kashwan who found specific-mutational patterns of *p53* gene in bladder transitional cell carcinoma among Iraqi patients exposed to war environmental hazards [16]. Young patients with cancer were also detected in another cancer types in Iraq such as leukemia [17, 18], breast cancer [19, 20] thyroid disorders [21, 22] and polycystic ovary syndrome[23,24] which put a high light on the environment situation in this country.

Subsequent studies demonstrated that *H-ras* mutations were more frequently observed in urinary tract tumors than the *K-ras* or *N-ras* genes [25]. Current results revealed that mutations in *K-ras* are more frequent in Iraqi bladder cancer patients than *H-ras* and *N-ras* (data not published yet). Other study was revealed that the most frequent *ras* mutations were *K-ras* G12D and *H-ras* Q61R. *K-ras* and *H-ras* mutations occurred with equal frequency, whereas *N-ras* mutations were not frequent in bladder cancer [26].

Mutations in the *ras* oncogenes (*H-ras*, *K-ras*, and *N-ras*) have been found in 13 % of bladder tumors and occurred in all stages and grades [27]. A study by Jebar and colleagues on 98 bladder tumors and 31 bladder cell lines, *ras* mutation was detected in 13 % of both types of samples [11].

In a total, there were 10 mutations in *H-ras*, 4 in *K-ras*, and 4 in *N-ras*. While this study showed different results, there were 5 mutations in *K-ras* region. Current result showed that compound *K-ras* mutations were existed in 3 patients and among 15 patients with *K-ras* mutations, 9 were smokers which suggest that carcinogens exist in cigarettes could be induce mutations in *ras* genes. Such correlation was established very well by others. Other study by [28] who stated that the numbers of smokers were 80 % and 16 % in patients and control subjects respectively, thus the smoking is risk factor for bladder cancer infection. Smoking is an established risk factor for bladder cancer [29]. Consistent with the epidemiological evidence for an association between bladder cancer and smoking, they found that about 51 % of patients were smokers, which shows a direct correlation between smoking and the incidence of bladder cancer [10]. Cigarette smoking substantially increases the risk of developing bladder cancer. Cigarette smoking duration is the major determinant for bladder cancer development risk independent of differences in tumor invasiveness or morphology [30].

Many previous studies have suggested an association between cigarette smoking and different types of cancers such as colon cancer or adenoma [31, 32]. Lung cancer [33, 34] and bladder cancer [35]. Smoking has been found to form many carcinogenic compounds which reach various tissues directly or indirectly. Carcinogens are known to cause genetic changes in multiple tumor suppressor genes and oncogenes resulting in cancer formation. Of these genes, *ras* family genes which are commonly mutated in human cancer including colon, stomach, lung, head and neck, bladder cancers and various other types of cancer. Tobacco carcinogens Increased frequency and altered spectrum of genes mutation have been observed in bladder cancer and lung cancer from not only smokers, but also non-smokers exposed to environmental tobacco smoke [35,36]. Tobacco smoking is the most well established risk factor for bladder cancer in both sexes [37, 38, 39]. The Population attributable risks for smoking was approximately 50 % in both sexes [40].

It has been estimated that smoking is a cause of 65 % of cancers overall, smokers have laryngeal cancer risks 10 times greater than non-smokers [41,42]. The risk increases as the frequency and duration of smoking levels increase [43, 44]. Cigarette smoking duration is the major determinant for bladder cancer development risk independent of differences in tumor invasiveness or morphology [45].

A dose-response relationship was observed between the numbers of cigarettes smoked per day and bladder cancer. An immediate and significant decrease in the risk of bladder cancer was also evident for those who quit smoking [46]. Cigarette smoking is the predominant risk factor for bladder cancer and is estimated to be responsible for 50 % of the cases in men, and 30 % of the cases in women [30] and found to be a rich source of reactive oxygen species that can induce a variety of DNA damage [47 ,48,49].

## 5. Conclusions

The prevalence of cancer is increasing in Iraq since 1980 and mutations in various detected genes associated with different type of cancers indicate a kind of exposing to carcinogens including those in cigarette smoking and other toxic hazards. The current results indicate that Iraqi population could be having instability in critical genes such c-oncogenes and tumor suppressor genes which lead to high frequency of cancer arising.



## **6. Recommendations**

We recommend to expand the study on large scale samples and gene expression to find the role of ras genes in bladder cancer arising.

## **Acknowledgement**

Authors would like to express their special appreciation and thanks to Ghazi Al Hariri and the National Al Amal hospitals in Baghdad-Iraq for their assistance in collecting samples from bladder cancer patients and other assists. Our thanks must go to the Institute of Genetic Engineering and Biotechnology-University of Baghdad, Iraq for great help in carrying all experiments and partial fund.

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