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Detection of Codon 12/13 g.6262G>A Mutation of *H-ras* Gene in Iraqi Bladder Carcinoma Patients

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Abstract: DNA was extracted from blood and urine samples from 45 patients with bladder carcinoma (age 20-87 years) in addition to samples from 25 apparently healthy persons as controls. Restriction fragment length polymorphism (RFLP) analysis was performed to determine genotypes of the *H-ras* codons12,13 using *Msp1* enzyme. The healthy results showed that two fragments (165 bp and 55 bp) were produce from the digestion with the enzyme for *H-ras* codon 12/13**.** These results indicated that the PCR amplified region of the codon 12/13 has one restriction site for the enzyme *Msp1*. The molecular analysis of the patient samples revealed that among 45 patients included in this study, 28 patients (62.2%) were with normal pattern (165 bp and 55 bp) and 17 patients (37.8%) were homozygous mutants (**g.6262G>A**). The frequency of g.6262 C>G mutation in patients was significantly higher than in apparently healthy subjects (37.3% versus 0%, OR= 0.033; X^2=0.966*, P<0.01).

Key words: Bladder carcinoma, *H-ras* , *MSP1* , RFLP, g.6262G>A

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Introduction

Bladder carcinoma is one of the most common cancer worldwide, it accounts for 6.5% of all cancers , with highest incidence in industrialized countries . It represents the fourth most common cancer in male and the eighth in female (1). Two main types of bladder carcinoma are identified : the transitional cell carcinomas (TCC) and the squamous cell carcinomas (SCC) where urinary schistosomiasis is an endemic disease. Rare types of bladder carcinoma include small cell carcinoma, carcinosarcoma, primary lymphoma and sarcoma (2). The name 'Ras' is an abbreviation of (Rat sarcoma), reflecting the way the first members of the protein family were discovered . The name *ras* is also used to refer to the family of genes encoding those proteins. When *ras* is 'switched on' by incoming signals , it subsequently switches on other proteins , which ultimately turn on genes involved in cell growth, differentiation and survival ,this can cause unintended and overactive signaling inside the cell , even in the absence of incoming signals (3). Because these signals result in cell growth and division, overactive *ras* signaling can ultimately lead to cancer (4) . The *ras* gene family consisting of 3 functional genes . *Harvey ras (H-ras)* , *Kristen ras (K-ras)* , and *Nuroblastoma ras* (*N-ras*) , encode highly similar and conserved proteins with a molecular weight of 21 kDa (p21) (5). The *H-ras* (Harvey rat sarcoma viral oncogene homologue)

proto oncogen is located at the terminal part of the short arm of chromosome 11. It consists of four encoding and one noncoding exon , the latter localized closer to the 5' end , a point mutation in one of the three hot spots (codons 12 , 13, and 61) may result in continuous stimulation of proliferation and development of many types of cancer (6,7) . Wide varieties of human cancer are found to have mutations in members of the *ras* gene family (8,9,10,11,12)**.** These mutations involved point mutations in codons (12, 13, or 61). These mutations lead to keep p21 in the GTP-bound in activate state' (13,14,15,16,17). Early detection of bladder carcinoma is extremely important and may have a major impact on the outcome because the early localized stage detection give 90% chance of surviving at least 5 years comparing to 9% chance of surviving in late detection (18,19,20). *H-ras* mutations are frequently observed in bladder cancer (21,22,23,24,25,26) which made screening of the *ras* mutations genes may be a useful marker for the early detection of bladder cancer (27,28,29)**.** The aim of this study was to investigate the diagnostic utility of the detection of *H-ras* mutations in blood and urine samples from the patients with bladder cancer, and to evaluate its potential as a diagnostic tool.

Materials and Methods

Blood and urine samples (3-5 ml) from 45 patients with bladder carcinoma and from 25 healthy (controls) were included in the study.

DNA samples were extracted from all samples using Bonier DNA extraction Kit (Korea). The concentration of DNA, purity and DNA integrity were determined by UV-spectrophotometry and agarose gel-electrophoresis. The codon 12/13 region of *H-ras* was amplified by PCR using the primers F-5-GGGCCGCAGGCCCCTGAGGA-3, R-5-CAGGGGCTGCAGCCAGCCCTAT-3 and the condition, initial denaturation 1 minutes at 96 ^{o}C, followed by 35 cycle each of denaturation 1 minute at 96 ^{o}C, annealing 1 minute at 69 ^{o}C, extension 1 minute at 72 ^{o}C and a final extension step at 72 ^{o}C for 7 minute. The PCR products were digested with *Msp1* enzyme and DNA sequencing.

Results and Discussion

The results of PCR analyses of *H-ras* codons 12/13 of the current study are shown in Figures 1 and 2. The PCR amplified regions of the *H-ras* codons 12/13 of healthy and patients showed a molecular weight of about 220 bp.

Restriction fragment length polymorphism (RFLP) analysis was performed to determine genotypes of the *H-ras* codons using *Msp1* enzyme. The healthy results showed that two fragments (165 bp and 55 bp) were produce from the digestion with the enzyme for *H-ras* codon 12/13 (Figures 3 and 4)**.** These results indicated that the PCR amplified region of the codon 12/13 has one restriction site for the enzyme *Msp1*.

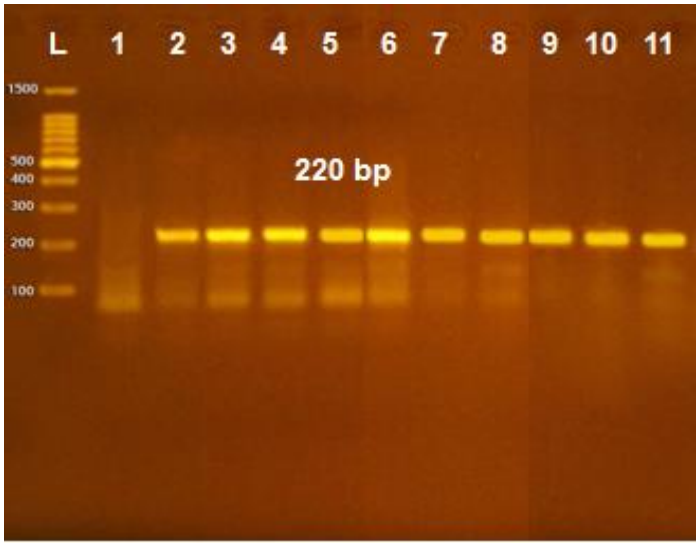

Figure 1: Gel electrophoresis of PCR products for healthy and patients with bladder cancer on 1% agarose using urine sediments extracted DNA .L , ladder 1,negative control ,2-8 patients bladder cancer ,9-11 DNA from a healthy person (220-bp)

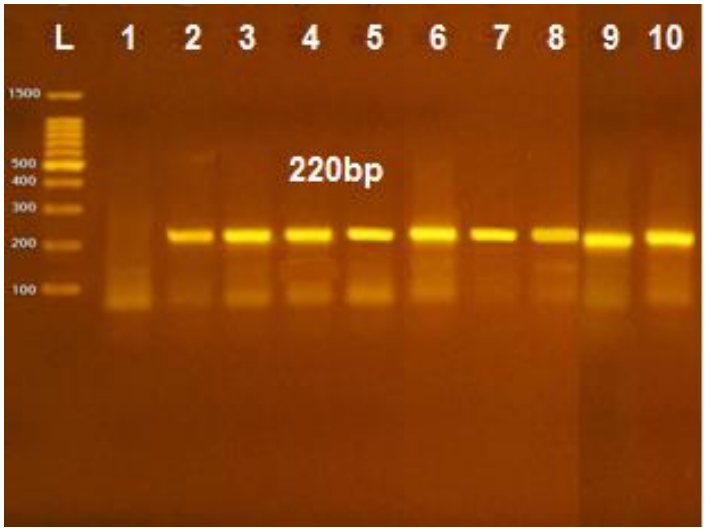

Figure 2 : Gel electrophoresis of PCR products for healthy and patients with bladder cancer on 1% agarose using blood extracted DNA .L , ladder 1,negative control ,2-8 patients bladder cancer ,9-10 DNA from a healthy person (220 bp)

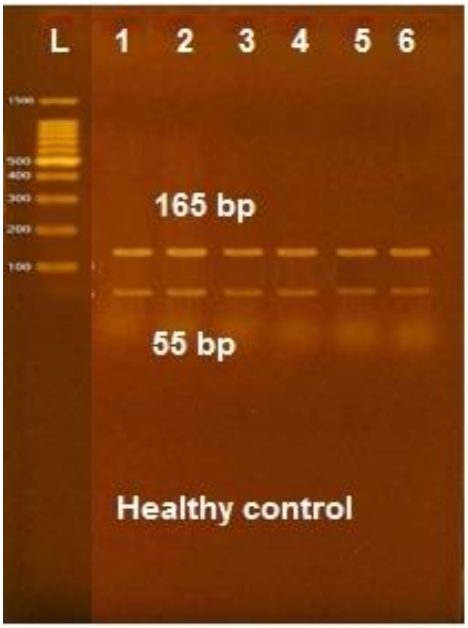

Figure 3: Gel electrophoresis of PCR products (*H-ras* 12/13 codons) for healthy digested with *Msp1* enzyme on 1% agarose. L, ladder; 1-6 DNA from healthy control

220 bp of the H-ras codons 12/13

1642 gggccgcaggcccctgaggagcgatgacgg
aatataagcggtggtggtg ggcg **cc…. 55 bp gg**cg gtgtgggcaa
gagtgcgctg accatccagc tgatccagaa ccattttgtg
gacgaatacg accccactat agaggtgagc ctagcgccgc
cgtccaggtg ccagcagctg ctgcgggcga gcccaggaca
cagccagg**at agggctggct gcagcccctg** 1861 **165 bp**

Figure 4: The restriction site ccgg of the enzyme *Msp1* among the 220 bp PCR products of the *H-ras* codon 12/13

The molecular analysis of the patient samples revealed that among 45 patients included in this study, 28 patients (62.2%) were with normal pattern (165 bp and 55 bp) and 17 patients (37.8%) were homozygous mutants (**g.6262G>A**) (Figure 5).

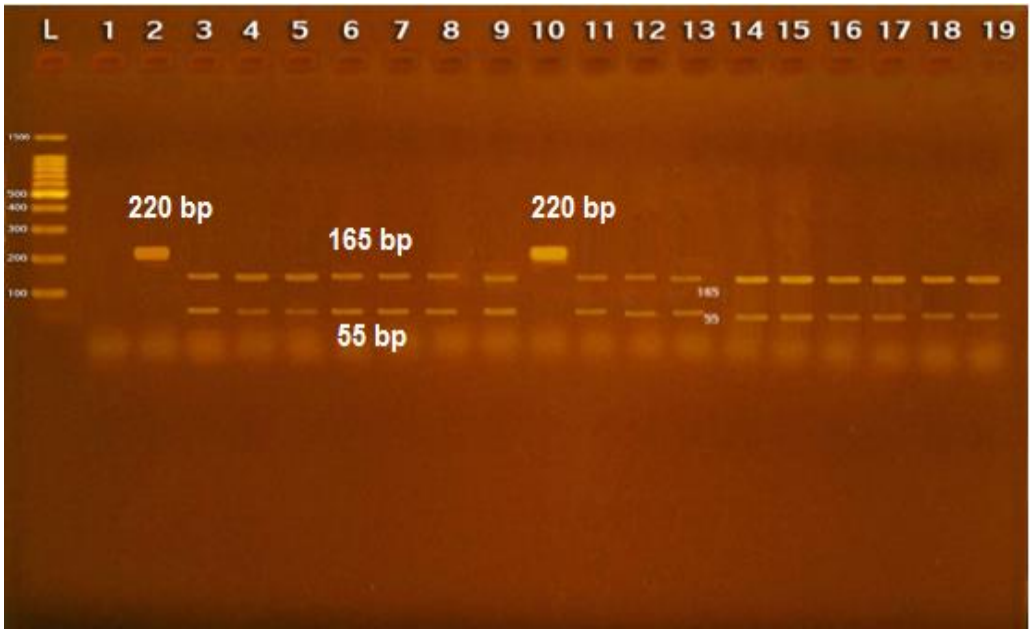

Figure 5 : Gel electrophoresis of PCR products (*H-ras* 12/13 codons) for patients with bladder cancer on 1% agarose. L, ladder ; 1,negative sample; 2 to 19, patients with bladder cancer. Patient samples 2 and 10 are with mutated *Msp1* site-undigested (220 bp)(g.6262G>A)

Genotype and Allele Frequency

The frequency of **g.6262G>A** mutation in patients was significantly higher than in apparently healthy subjects (37.3% versus 0%, OR= 0.033; X^2=0.966*, P<0.01). PCR-RFLP results of **g.6262G>A** mutation in codon 12/13 region of *H-ras* gene is presented in table 2. The results showed that 28 (62.2%) patients with CC genotype have bladder cancer which indicate that cancer initiated by another reason than **g.6262G>A** mutation in codon 12/13. On the other hand, high significant differences between control and patient groups were observed for GG genotype and for allele frequency which indicate association with bladder cancer. However, the risk of having cancer due to this mutation is low.

Table 2 : Distribution of genotype frequencies of the *H-ras* g.6262G>A polymorphisms in patients and control

Site /Exon2 6206-6425 NG-007666.1(220 bp)	Control	Patient	O.R.	Chi-square X^2	g.6262G>A
Genotype CC	25(100%)	28(62.2%)	0.617	1.027NS	Homozygous 17
CG	0(0.0%)	0(0.0%)	0.00	0.00	Heterozygous 0
GG	0(0.0%)	17(37.8%)	0.033	0.966*	
P-value	0.0014**	0.0025**	---	----	
Allele Frequency					
C	50(100%)	56(62.2%)	0.617	1.027NS	
G	0(0%)	34(37.8%)	0.033	0.966*	
**P< 0.01 , NS: Non Significant					

All mutations which were detected in our bladder cancer patients were detected in their blood and urine samples which make urine samples very important for diagnosis. Diagnosis of bladder cancer using examination of urine is good but the sensitivity of this technique is very low (30). Urine usually contains a mixture of cells with a numbers of genetically-normal and abnormal epithelial and white blood cells. Since only a small fraction of the cells may contain the mutation, then detection of the *ras* mutation requires a sensitive assay (9). Because of their remarkable sensitivity, PCR-based techniques showed to be suitable to detect mutations in cells in the urine of patients with bladder cancer where all mutations detected in DNA extracted from blood sample of bladder cancer patients were detected in DNA extracted from urine sediments epithelial cells which suggest that urine offers a useful sample for mutations detection in bladder carcinoma. The results obtained by the current study showed that 17(37.8%) of the patients were with homozygous mutants to the mutation (**g.6262G>A**) of the codon 12/13. The *H-ras* mutation was first detected in the human bladder carcinoma cell line T24 (31,32) Subsequent studies demonstrated that *H-ras* mutations were more frequently observed in urinary tract tumors than the *K-ras* or *N-ras* genes (33). Altogether, findings indicate that the detection of *H-ras* mutations in urine sample (as an adjunct to a cytologic examination) may substantially improve the sensitivity of detecting bladder carcinoma.

The previously reported frequency of mutations in H-*ras* codon 12 ranges from 3% to 76%. Although most authors have found a frequency around 20% (34,35,36). The frequency of cancers with codon 12 mutations of the *H-ras* was high at 37.8% (17 patient of 45), this being comparable to the 36.36% frequency reported by (37) and

less than those detected by (38). Their results showed that *H-ras* mutations in 12 of 13 was 92.3% in tumor tissues and 11 of 13 (84.6%) in urine samples from patients with superficial bladder carcinoma. Other studies detected between 13% to 45% of *H-ras* mutations in urine sediments from bladder carcinoma patients (38,39). Recent study showed that *H-ras* LOH was simultaneously associated with *P53* &*Rb1* in some cases and that there is no correlation between tumor grade and stages with *H-ras* mutations (40). Mutations in the *ras* oncogenes (*H-ras*, *K-ras*, *N-ras*) have also been found in 13% of bladder tumors and occurred in all stages and grades (41). Mutation status of *ras* in the tumor has important clinical implications as it may affect the response to treatment and has treatment-independent prognostic value (37,42,43). It is estimated that 20% of all tumors have undergone an activating mutation in one of the three *ras* genes (13). Although new tests for cancer antigens in blood or urine have been developed, their accuracy and sensitivity is distant from ideal (19). Somatic mutations in the *H-ras*, *K-ras* and *N-ras* genes in bladder carcinoma affect codons 12, 13 and 61.These mutations frequently coincided with FGFR3 mutations. A new study on the expression of *H-ras* in 48 pTa bladder carcinoma showed an inverse correlation of expression value with recurrence and progression (44).

The genotype and allelic association with bladder cancer were observed by other researcher. The frequency of the codon 12 mutation was 37.8% in the present study. This is similar to other studies (10,25,32), but less than those detected by several reports on the bladder (12,40). The homozygous genotype of some mutations of the *H-ras* proto-oncogene are detected at an increased risk of bladder cancer (45,46,47). Interestingly, these mutations found to associate with combined high grade and advanced tumor (10).

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