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Detection of Three Novel Mutations in Exon 7 of FGFR3 Gene in 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 October 2013 to October 2014, for detecting the role of genetic alterations of *FGFR3* gene in Iraqi patients with bladder cancer. 50 patients with bladder cancer who admitted to Ghazi AlHariri Hospital in Baghdad and 25 healthy persons (age between 30 to 86 years) were included in this study. Total genomic DNA was isolated from blood samples for molecular analysis using specific primers for exon 7 of the gene *FGFR3*. The PCR amplified regions of the *FGFR3* exon 7 of healthy and patients showed a molecular weight of about 120 bp. The analysis of mutation using restriction fragment length polymorphism (RFLP) was performed on PCR products of *FGFR3* exon 7 using *Hae* II and *Tse*I enzymes. These results showed that the PCR amplified regions of the *FGFR3* exon 7 has only one restriction site for each enzymes. The REFLP molecular analysis of *FGFR3* exon 7 of patient samples for both enzymes revealed one mutation in one patient include *FGFR3* Arginine 248 Cysteine mutation. The DNA sequencing analysis of the exon 7 PCR products revealed that among 50 patients included in this study, 51 mutations were detected in exon 7. The mutations detected in exon 7 include three types, g.13515 del C , g.13510 del A and g.13529 ins A. The more frequent mutation was g.13515 del C which detected in 34 patients followed by g.13510 del A and g.13529 ins A mutations which detected in 12 and 1 patients respectively. The A insertion mutation (13529) were included in the *Hae II* restriction site which explain the single mutation detected in patients. The results showed that the exon 7, g.1315 delC mutation is a correlated with the initiation of tumor since it detected in all grads and consist of the majority of detected mutations (36/81, 44.4%). On the other hand, exons 7 mutations, g.13529 ins A, g.13510 del A, showed to have importance in cancer initiation and development since they are detected in the early grade (G1) and in 38(80.9%) patients of 47.
Key words: Bladder carcinoma, FGFR3, RFLP, g.1315 delC, g.13529 ins A, g.13510 del A

Introduction:

Bladder cancer is the ninth most common cancer diagnosis worldwide, with more than 330,000 new cases each year and more than 130,000 deaths per year, with an estimated male:female ratio of 3.8:1.0 (Ploeg, 2009). Approximately 90% of malignant tumors arising in the uroepithelium of the bladder are transitional cell carcinomas (TCC). It starts in the inner layer of the bladder (Siegel, 2012).

This group has subtypes: Papillary cancers grow like tiny fingers from the inner bladder lining toward its hollow center, Flat cancers do not grow toward the center, These tumors are also named based on whether they have grown into the bladder wall, Non-invasive cancers are still in the inner layer of cells (the urothelium) but have not grown into the deeper layers (Lopez 2004), and Invasive cancers have grown into the deeper layers of the bladder. These cancers are more likely to spread and are harder to treat (Arianayagam, 2011).

Other histologists include squamous cell carcinoma (1.5%), This type is much less common and is usually invasive. adenocarcinoma (1.2%), This type is also much less common, almost all are invasive, respond poorly to radiation with chemotherapy, and small cell carcinoma (<1%), A very small number of bladder cancers are of this type. These cancers often grow quickly (Zhang *et al*., 2012). Sarcomas start in the muscle cells of the bladder, but they are rare (Bodoor *et al* 2010), to find out more about sarcomas (Arianayagam and Rashid, 2011**).** Most superficial bladder tumors show a loss of heterozygosity (LOH) of chromosome 9 (Wada *et al.*,2003). deletions on chromosome 9 are the most common chromosomal abnormalities in transitional cell carcinoma (TCC), which represents more than 50% of all grades and stages (Miyao *et al*., 1993). low-grade papillary tumors and LOH of chromosome 9 exhibit a constitutive activation of the receptor tyrosine kinase-Ras pathway, with activating mutations in the *HRAS* and fibroblast growth factor receptor 3 (Mitra *et al.,* 2006). *FGFR3* mutations are confined to hot spots in exons 7, 10, and 15, and all are predicted to cause constitutive activation of the kinase activity of the receptor, which in turn can activate the mitogen-activated protein kinase (MAPK) pathway—a pathway shared with the Ras family of proteins (Mitra *et al.,* 2006). Mutations in *FGFR3* and the Ras genes (including *HRAS*), both mutations were found to be absolutely mutually exclusive, suggesting possible biological equivalence (Jebar *et al.,* 2005).

Approximately 80% of transitional cell carcinomas are confined to the epithelium (pTa, Carcinoma in situ) or lamina propria (pT1) at initial diagnosis, but the remaining 20% invade the muscularis propria (pT2, pT3, pT4). pTa lesions (papillary tumors) are the most common form of bladder carcinoma (Billerey *et al*., 2001).

More than 50% of primary bladder urothelial cell carcinomas, especially in low-grade and low-stage papillary tumors (Hernandez *et al.,* 2005).

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According to the most recent (Iraqi cancer registry, 2010), bladder carcinoma is currently ranks sixth among the commonest ten cancers. The male gender seems to bear this problem than females in that it is the second most frequent cancer in males (827 cases) and Ninth position in females (274 cases) (Iraqi cancer registry, 2010).

This research aims for the detection of three novel mutations in exon 7 of *FGFR3* gene in Iraqi patients with bladder cancer.

Materials and Methods

The study consisted of 50 subjects with bladder cancer (Transitional cells carcinoma TCC) and 25 subjects control group. Patient samples were obtained from Ghazi Al Hariri Hospital in Baghdad. Patients age ranged from 30 to 86 years while control subjects ages ranged from 30 to 50 years. Blood samples (3-5ml) were collected from patients and control in EDTA anticoagulant tubes. ***Questionnaire form was filled for each patient including; name, age, gender, employment type and smoking.***

DNA extraction

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

Polymerase chain reaction (PCR) for exon 7(120bp)

The exon 7 region of *FGFR3* was amplified by PCR using the primers, F 5' CGGCAGTGGCGGTGGTGGTG'3 and R 5' AGCACCGCCGTCTGGTTG '3 and the condition, initial denaturation 5 minutes at 95 ^{o}C, followed by 40 cycle each of denaturation 1 minute at 95 ^{o}C, annealing 1 minute at 67 ^{o}C, extension 1 minute at 72 ^{o}C and a final extension step at 72 ^{o}C for 10 minute. PCR products (3 µl) were then separated on 3% agarose gel with a ladder (100bp) and visualized.

Screening for mutations in exon 7 using RFLP analysis

Screening of the *FGFR3* mutations in exon 7 was performed by digestion with restriction endonuclease *Hae*II (New England Biolab Beverly, MA, USA) and *Tse*I (New England Biolab). The PCR product (10 µL) was digested with 1 U of each restriction enzyme in 20 µL for 1 h at 37°C for *Hae*II and at 65°C for *Tse*I. Each product was analyzed by 3% agarose gel then photographed under ultraviolet light.

PCR products Sequencing

The PCR products of the *FGFR3* gene exon 7 region (50 samples) and primers were sent by Macrogen Company (U.S.A) for sequencing. The sequences of these samples were compared with the information in gene bank of the National Center for Biotechnology Information (NCBI) for standard *FGFR3* gene, using (Mega -6) software.

Results and discussion

Subjects data:

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

Table 1: Bladder cancer and healthy profiles.

Characteristics	Healthy control	Patients
Age(years)		
Mean	**58**	**62**
Range	**30-50**	**30-86**
Male	**23**	**46**
Female	**2**	**4**
Smoking		
Yes	**13**	**39**
no	**12**	**11**
Family history	**0**	**0**

Bladder cancer is rare in people younger than 50 years of age, even though it can occur at any age (Parag *et al*., 2009; Dodurga, *et al.,* 2011). The incidence of cancer increases directly with age with the median age at diagnosis of around (70) years for each gender (Lynch and Cohen, 1995). It has been observed that genetic alterations that are frequently seen in older adults are extremely rare in young patients. Urothelial neoplasms in children and young adults appear to be biologically distinct and lack genetic instability in most cases (Wild *et al.,* 2007).

REFLP analysis of *FGFR3* exon 7

PCR analysis of *FGFR3* exon 7 is shown in Figure 1.

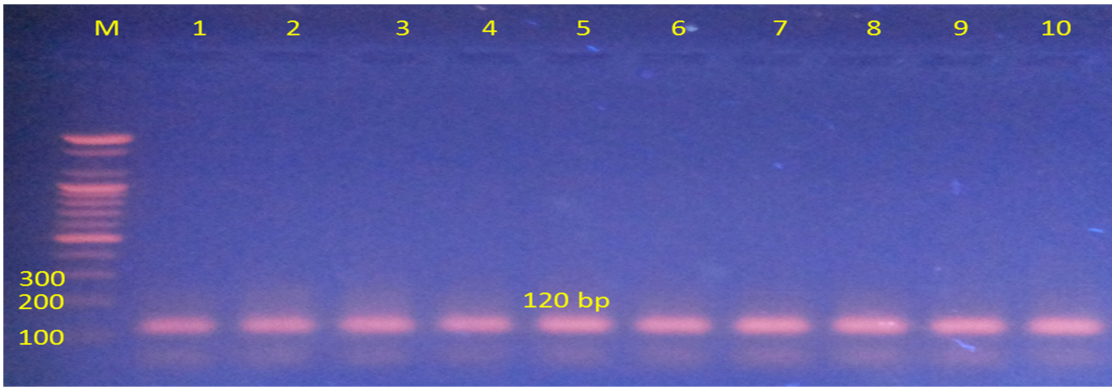

Figure (1): Gel electrophoresis of *FGFR3* exon 7 PCR products for healthy and patients with bladder cancer on 3% agarose stained with Ethidium Bromide and visulized under U.V light using blood extracted DNA. M, ladder, 1-7 patients bladder cancer samples, 8-10 healthy control samples.

The analysis of mutation using restriction fragment length polymorphism (RFLP) was performed on PCR products of *FGFR3* exon 7 using *Hae* II and *Tse*I enzymes. If there were no mutations, a 120 bp exon 7 PCR product will be digested by *Hae*II enzyme to 64- and 56-bp fragments and digested to 94 and 26 bp fragments by *Tse*I enzyme, as follow and as in figures 2 and 3:

***FGFR3* exon 7 region/ *Hae*II enzyme site**

13465CGGCAGTGGCGGTGGTGGTGAGGGAGGGGGTGGCCCCTGAGCGTCATCTGCCCCCACA GAGCGC$^{\nabla}$TCCCCGCACCGGCCCATCCTGCAGGCGGGGCTGCCGGCCAACCAGACGGCGGTG CT 13584.

***FGFR3* exon 7 region/ *Tse*I enzyme site**

1365CGGCAGTGGCGGTGGTGGTGAGGGAGGGGGTGGCCCCTGAGCGTCATCTGCCCCCACAGAG CGCTCCCCGCACCGGCCCATCCTGCAGGCGGG**G$^{\nabla}$CTGC**CGG **CAACCAGACGGCGGTGCT 13584.**

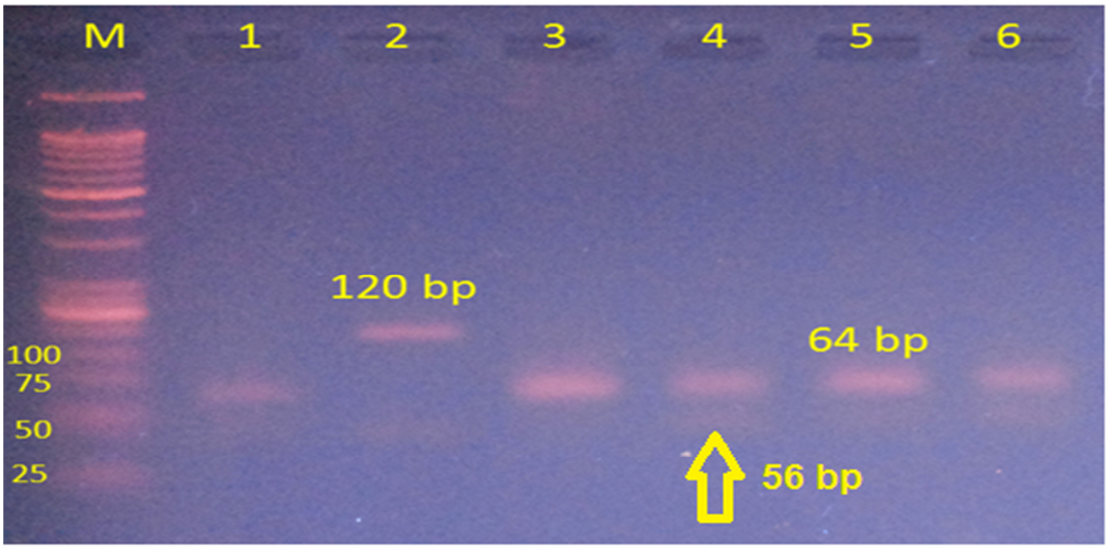

Figure (2): Gel electrophoresis of PCR products (*FGFR3* exon 7) for healthy digested with *Hae* II enzyme on 3% agarose. M, ladder; 1, 3, 4, 5, 6: DNA from healthy control, lane 2: PCR undigested product.

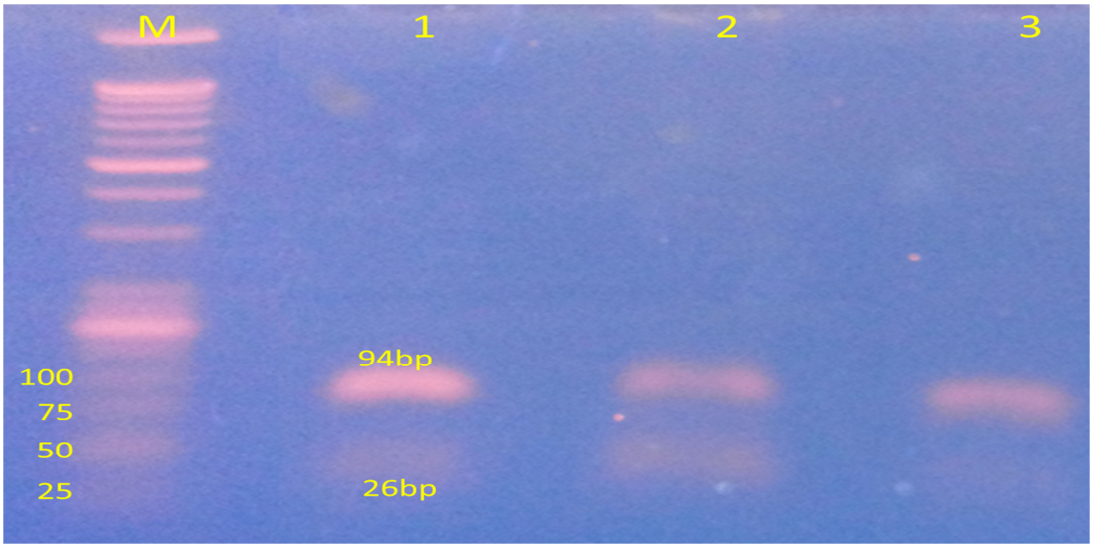

Figure (3): Gel electrophoresis of PCR products (*FGFR3* exon 7) for healthy digested with *Tse*I enzyme on 3% agarose. M, ladder; 1, 2, 3: DNA from healthy control.

These results showed that the PCR amplified regions of the *FGFR3* exon 7 has only one restriction site for each enzymes. The REFLP molecular analysis of *FGFR3* exon 7 of patient samples for both enzymes revealed one mutation in one patient include *FGFR3* Arginine 248 Cysteine mutation.

Detection of *FGFR3* exon 7 mutations by DNA sequencing

The bladder cancer patients PCR products of the exon 7 and of the *FGFR3* gene were screened for mutations by sequencing. The patients sequencing results were compared with human reference *FGFR3* gene sequence (http:NCBI Reference Sequence).

Among 50 patients included in this study, 47 (94%) patients were with FRGF mutations. The total number of detected mutations were 51 (63%) mutations. The mutations detected in exon 7 include three types, g.13515 del C , g.13510 del A and g.13529 ins A. The more frequent mutation was g.13515 del C which detected in 34 patients followed by g.13510 del A and g.13529 ins A mutations which detected in 12 and 1 patients respectively. The A insertion mutation (13529) included in the *Hae II* restriction site which explain the single mutation detected in patients (Table (2)(Figures 4, 5, and 6). All mutations in the tables are considered novel and not registered in the NCBI.

Table (2): Mutation analysis of the exon 7

Exone 7			
Mutation	No. of mutation in 47 patients / %	Change code	Effect
g.13515 del C GCC>G-C	34 / 72 %	Cys/Cys	Framshift
g.13510 del A TCA>TC-	12 / 30%	Ser/Ser	Framshift
g.13529 Ins A TCC>ATC	1 / 2%	Ser/Ile	Framshift

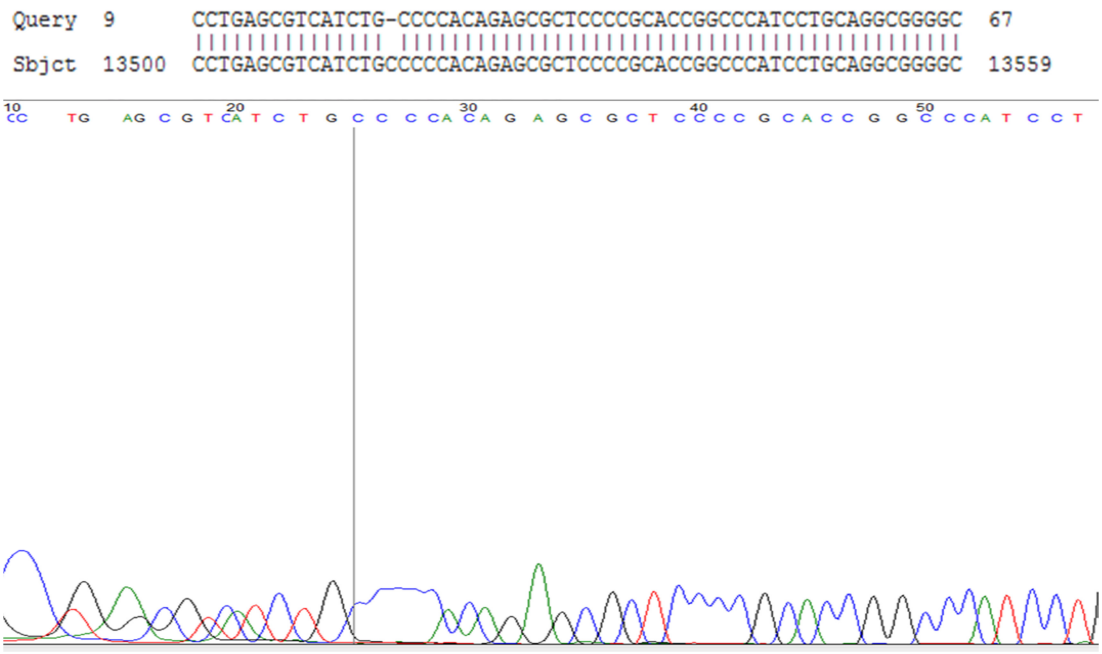

Figure (4): Site 13515 del C, nucleotide sequence (forward) in exon 7.

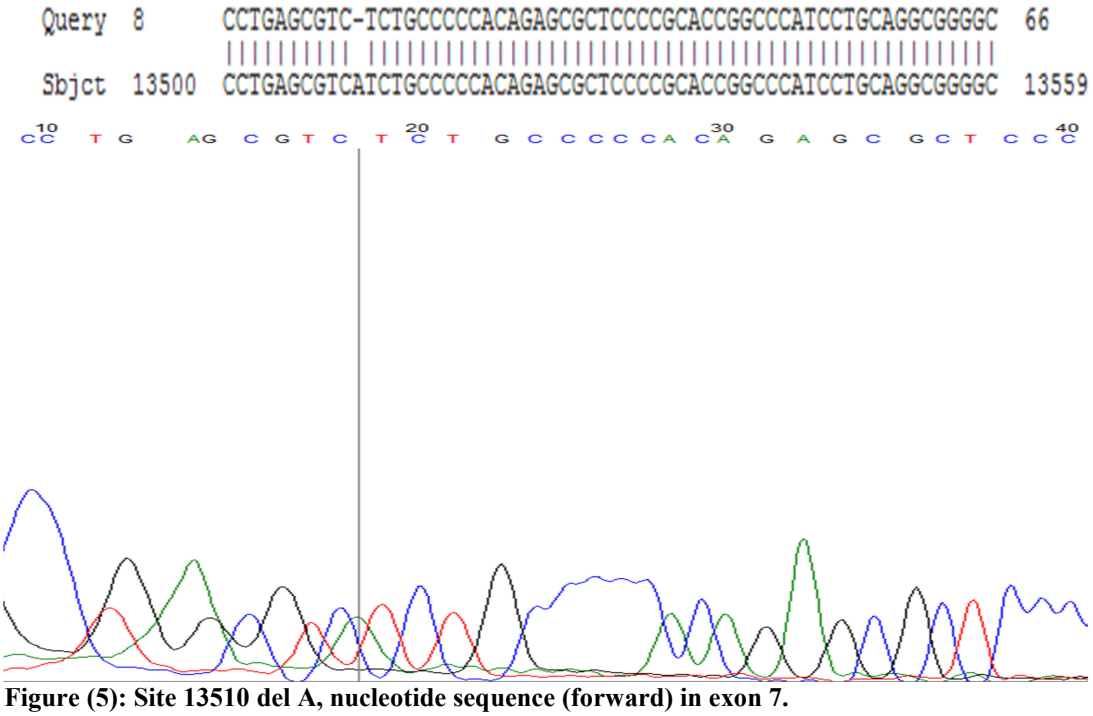

Figure (5): Site 13510 del A, nucleotide sequence (forward) in exon 7.

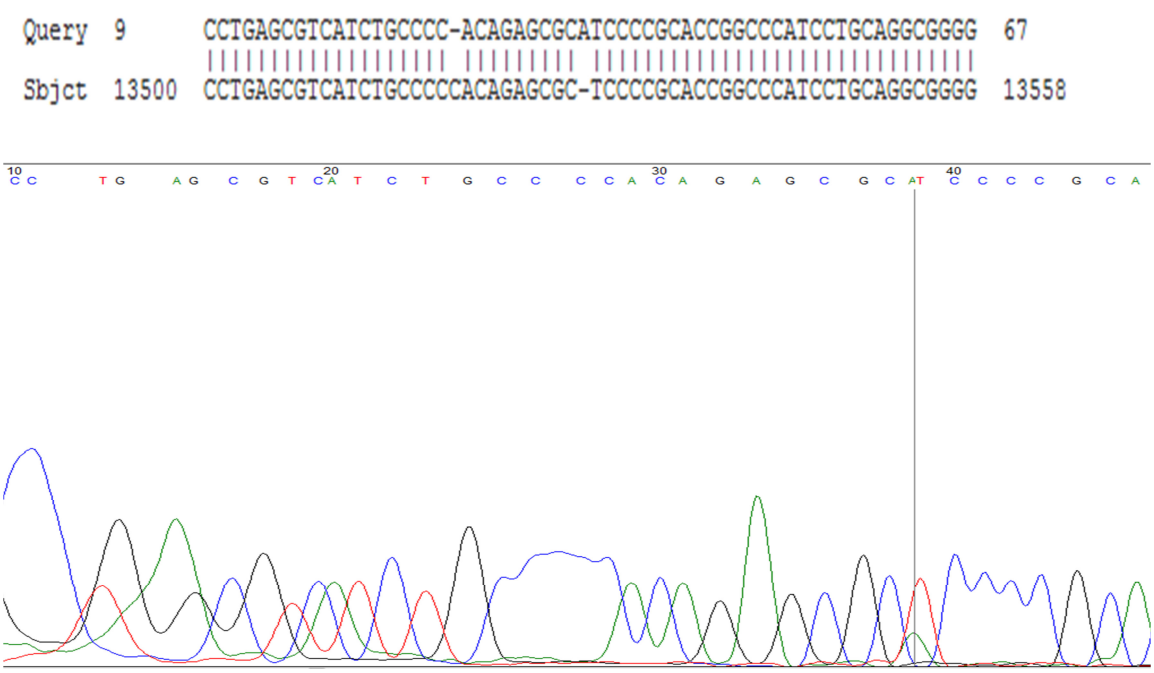

Figure (6): Site 13529 Ins A, nucleotide sequence (forward) in exon 7.

In this study, The REFLP molecular analysis of *FGFR3* exon 7 of patient samples for both enzymes revealed one mutation in one patient include *FGFR3* Arginine 248 Cysteine mutation and on mutation at Serine 249 Cysteine.

Mutations of the codons 248,249 and 372 of the *FGFR* 3 exon 7 were also detected in bladder cancer patients by several researchers (Cappellen *et al*., 1999; Passos *et al*., 1999; Ashraf and Herve, 2003). Other exon 7 mutations were also reported (Johanna *et al*., 2005; Hernandez *et al*., 2006 ; Junker *et al*.,2008). *FGFR3* gene mutations in bladder cancer patients were also detected by Dodurga *et al*.(2011) who detect mutations in the exon7 of the gene *FGFR* 3 in 12% of bladder cancer patients include Arginine 248 Cysteine and in 50% of the bladder cancer patients include Serine 249 Cysteine. *FGFR* 3 exons 7 mutations were also detected in bladder cancer patients by Takahiro *et al*. (2001) who detect 13 mutations include Arginine 248 Cysteine and Serine 249 Cysteine of the exon 7.

The association between bladder cancer grad and FGFR3 mutations

Our results showed that there is a good correlation between the development of bladder cancer and FGFR3 mutations (Table 4). The results showed that the exon 7, g.1315 delC mutation is a correlated with the initiation of tumor since it detected in all grads and consist of the majority of detected mutations (36/81, 44.4%). On the other hand, exons 7 mutations, g.13529 ins A, g.13510 del A, showed to have importance in cancer initiation and development since they are detected in the early grade (G1) and in 38(80.9%) patients of 47.

Table (3): The number of mutations of the *FGFR3* Exon 7 in different cancer grads.

Mutation / Grade	g.13515 del C	g.13515 del C	g.13529 ins A	Total
G1	12	8	3	23
G2	12	2	-	14
G3	12	2	-	14
Total	36	12	3	51

Previous studies indicated a strong correlation between FGFR3 mutations and the stage/grade of the tumor (van Rhijn *et al* 2001; Hernandez *et al*., 2006). No significant correlation between *FGFR3* expression and stage or grade of the tumor (Matsumoto *et al*., 2004).

In a study by (Khaldon *et al.,* 2010) find a significant correlation ($p < 0.001$) between the stage of the tumor and *FGFR3* mutations. However, in contrast to other studies, they could not find any correlation between the grade of the tumor and *FGFR3* mutations. FGFR3 protein expression of moderate or high levels of protein in 49% of tumors but found no relationship to tumor grade or stage. Two other studies found a relationship between higher expression and lower tumor grade and stage (Gomez *et al.,* 2005). Some of the studies have reported that low-stage and low-grade bladder tumors are associated with *FGFR3* mutations (Van Tilborg *et al*., 2002; Hirao *et al*., 2005). The high frequency of *FGFR3* mutations in pTa tumors, and their low frequency in pT1 and pT2–4 tumors are consistent with the model of bladder tumor (Lee and Droller, 2000; Knowles, 1999). *FGFR3* mutations were detected at (21% pT1), (16% pT2 to pT4), twenty-seven from thirty-two (84% G1), Sixteen from

twenty-nine (55% G2) and five from twenty-one (7% G3) (Claude *et al*., 2001). FGFR3 protein expression of moderate or high levels of protein in 49% of tumors but found no relationship to tumor grade or stage. Other studies found a relationship between higher expression and lower tumor grade and stage (Gomez, *et al.,* 2005). Other studies revealed that no mutations of the *FGFR3* gene in bladder tumor were reported (Tomlinson *et al.,* 2007; Arshad *et al*., 2010).

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