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Association of Exon 9 *FGFR3* Mutations and Cancer Grads in Patients with Bladder Cancer

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Abstract: 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. According to the most recent Iraqi cancer record (Iraqi cancer registry, 2010), bladder carcinoma is currently ranks sixth among the commonest ten cancers. Previous studies indicated that genetic alterations were involved in bladder cancer.

To detect genetic alteration in exon 9 of ***FGFR3*** gene, 50 patients with different grads of bladder cancer who admitted to Ghazi AL-Hariri Hospital in Baghdad and 25 healthy persons aged between 30 to 86 years were included in this study. DNA was extracted from blood samples from patients and healthy control. PCR was conducted using special primers. Mutations of exon 9 of the *FGFR3* gene were screened by sequencing and the patients sequencing results were compared with human reference *FGFR3* gene sequence (**NCBI Reference Sequence: NG_012632.1**).

Among 50 patients included in this study, 30 (37%) patients were with mutations detected in exon 9 which include novel g.16026 del G and g.16024 sub G>C mutations. The more frequent mutation was g.16026 del G (22, 70%) followed by g.16024 sub G>C mutation (8, 30%). Moreover, the results showed that three patients were with compound mutations (with both exon 9 mutations).

Exon 9 mutations (g.16024 sub G to C and g.16026 del G) showed an association with cancer initiation and metastasis since they detected in grad I and II.

Key words: Bladder cancer, *FGFR3*, Mutations, g.16026 del G, g.16024 sub G>C.

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Introduction

There are two major isoforms of *FGFR3* transcripts that are generated from a mutually exclusive splicing event in which the second half of the third Ig-like domain is encoded by either the 151 nucleotides of exon 8 (FGFR3b) or the 145 nucleotides of exon 9 (FGFR3c) (1). FGFR3b is the main form found in epithelial cells, whereas FGFR3c is the predominant form found in mesenchyme cells (2). The *FGFR3* gene provides instructions for making a protein called fibroblast growth factor receptor 3 (3). This protein is part of a family of four fibroblast growth factor receptors that

share similar structures and functions (4). These proteins play a role in several important cellular processes, including regulation of cell growth and division, determination of cell type, formation of blood vessels, wound healing, and embryo development (5). The FGFR3 protein spans the cell membrane, so that one end of the protein remains inside the cell and the other end projects from the outer surface of the cell (6). This positioning of the protein allows it to interact with specific growth factors outside the cell and to receive signals that control growth and development. When these growth factors attach to the FGFR3 protein (7), the protein is turned on (activated), which triggers a cascade of chemical reactions inside the cell that instruct the cell to undergo certain changes, such as maturing to take on specialized functions (8).

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 (9). 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 (10). According to the most recent data (11), 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).

The major genes involved in bladder cancer are *FGFR3* and *H-ras*. Mutations in *FGFR3* and the ras genes (including *H-ras*), both mutations were found to be absolutely mutually exclusive, suggesting possible biological equivalence (12). This mutual exclusion suggests that *FGFR3* and ras gene mutations may represent alternative means to confer the same phenotype on bladder-cancer cells wherein oncogenic activation of either *FGFR3* or *HRAS* results in stimulation of the mitogen-activated protein kinase (MAPK) pathway (13). *FGFR3* mutations are confined to hot spots in exons 7, 9, and 15, and all are predicted to cause constitutive activation of the kinase activity of the receptor, which in turn can activate the (MAPK) pathway—a pathway shared with the ras family of proteins (14). 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 (15). Mutations have been described to be more frequent in low grade or papillary superficial tumors and to decrease with stage and grade (16,17). This is thought to be due to the emergence of tumors following a different molecular pathway with no FGFR3 mutations in more invasive tumors. *FGFR3* mutations have also been observed in patients with favorable disease characteristics (18,19), *FGFR*3-mutated tumors have malignant potential. Mutations have shown to fail to predict the risk of recurrence and progression in a small group of superficial and invasive tumors (20), *FGFR*3 mutations have been associated with recurrence in TaG1 bladder tumors (21). FGFR 3 overexpression has been shown to be more frequent in low grade and stage, and not correlated to recurrence and progression (22).

The aim of this study is to find the association between grades of bladder cancer and *FGFR*3 mutations.

Materials and Methods

The study consisted of 50 subjects with bladder cancer (Transitional cells carcinoma TCC) and 25 subjects control group. Patients blood 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 9 (270bp)

The exon 9 region of *FGFR3* was amplified by PCR using the primers, F 5' CAGGCCAGGCCTCAACGCCC '3 and R 5'AGGCCTGGCGGGCAGGCAGC '3 with 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 72 ^{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 2% agarose gel with a ladder (100bp) and visualized.

PCR Products Sequencing

The PCR products of the *FGFR3* gene exon 9 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, grade and stage.

Table (1): Characteristics of bladder cancer patients group

Characteristics	Tumor stage				Chi-square
	a	1	2	3	
No.	14	21	12	3	
Grade : 1	4 (28.57%)	3 (14.29%)	2 (16.67%)	0 (0.0%)	9.54 **
Grade : 2	6 (42.86%)	8 (38.10%)	4 (33.33%)	2 (66.67%)	11.73 **
Grade : 3	4 (28.57%)	10 (47.62%)	6 (50.0%)	1 (33.33%)	8.92 **
Chi-square	5.24 *	9.53 **	9.81 **	13.67 **	--
Age (year)					
Mean	52	60	63	68	--
Range	30-70	40-80	43-74	47-87	--
Gender					
Male	13 (92.86%)	19 (90.48%)	11 (91.67%)	3 (100.0%)	4.68 *
Female	1 (7.14%)	2 (9.52%)	1 (8.33%)	0 (0.00%)	4.68 *
Chi-square	14.37 **	13.75 **	13.92 **	15.00 **	--

In the present study there was no correlation between the occurrence of bladder cancer and patient's age, gender or family history.

Previous studies indicated no significant correlation between the occurrence of bladder cancer and patient's gender. The incidence of various cancer stages and grades was not statistically different among both the gender groups. Bladder cancer is rare in people younger than 50 years of age, even though it can occur at any age (23, 24). The incidence of cancer increases directly with age with the median age at diagnosis of around (70) years for each gender (25). Of the 216 cases of bladder cancer, 183 (84.7%) had a history of hematuria with no significant differences in age distribution between sexes (26). Younger individuals present more frequently with low-grade and low-stage tumors than their elderly counterparts (27) and behave in an indolent fashion (28). This is contrary to the common belief in malignancy that biological behavior of a cancer is more aggressive in younger age groups. This fact is underplayed in the literature and research should be carried out to find out the reasons for this. However, 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 (29).

PCR Analysis of *FGFR3* Exons 9

PCR analysis of *FGFR3* exon 9 is shown in (Figure 1).

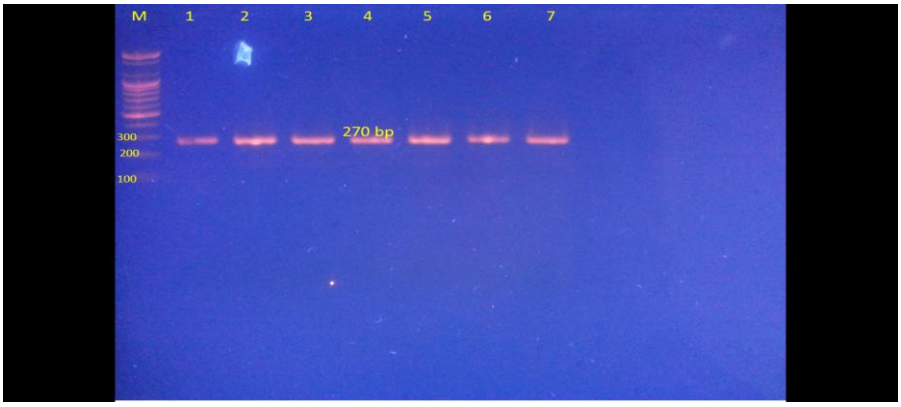

Figure (1): PCR products for FGFR3 exon 9 on agarose gel (2%) after electrophoresis for 1 hour at 100 volt. M: DNA ladder marker (100 bp) lane (1) to (5) for blood samples of bladder cancer patients. Lane (6) to (7) for blood samples of healthy controls

On the other hand, no mutation was detected in *FGFR3* exons 9 since no restriction sites were exists for both enzymes (***Hae*II enzyme site** AGCGCT, ***Tse*I enzyme site** GCTGC) (Figure 2).

15987CAGGCCAGGCCTCAACGCCCATGTCTTTGCAGCCGAGGAGGAGCTGGTGGAGGCTGACGA GGCGGGCAGGTGTATGCAGGCATCCTCAGCTACGGGGTGGGCTTCTTCCTGTTCATCCTGGTGG TGGCGGCTGTGACGTCTGCCGCCTGCGCAGCCCCCCCAAGAAAGGCCTGGGCTCCCCCACCGTG CACAAGATCTCCCGCTTCCGCTCAAGCGACAGGTAACAGAAAGTAGATACCAGGTTCTGAGCTGC CTGCCCGCCAGGCCT16257.

Figure (2): The sequence of the *FGFR3* exon 9 PCR product according to NCBI Ref

Detection of *FGFR3* Exons 9 mutations by DNA Sequencing

Thirty mutations were detected in exon 9 which include two types, g.16026 del G and g.16024 sub G>C mutations. The more frequent mutation was 16026 del G (21, 70%) followed by 16024 sub G>C mutation (9, 30%). All mutations in the table (2)(Figures 3 &4) are considered novel and not registered in the NCBI.

Table (2): Mutations analysis of the exon 9

Exon 9					
Mutation	No. of mutation in 50 patients (%)	Change code	Effect	X^2	OR
g. 16026 del G GAG>GA-	21/70 (30.00%)	Glu/Glu	Frame shift	8.50**	1.09
g.16024 sub G GAG>CAG	9/30 (30.00%)	Glu/Gln	Missense	12.25**	1.48
** (P<0.01).					

The frequency of g.16026 and g.16024 mutations was high significantly ($p<0.01$) with (OR) odd ratio 1.09 and 1.48 respectively. Exon 9 mutations were also identified in bladder cancer patients by others. More than 15 type of mutations were reported in exon 9 (8,16,30). The more frequent mutations among these mutations are mutations of the codons 372,373,375 and 382 which include Glycine 372 Cysteine and Tyrosine 375 Cysteine (31).

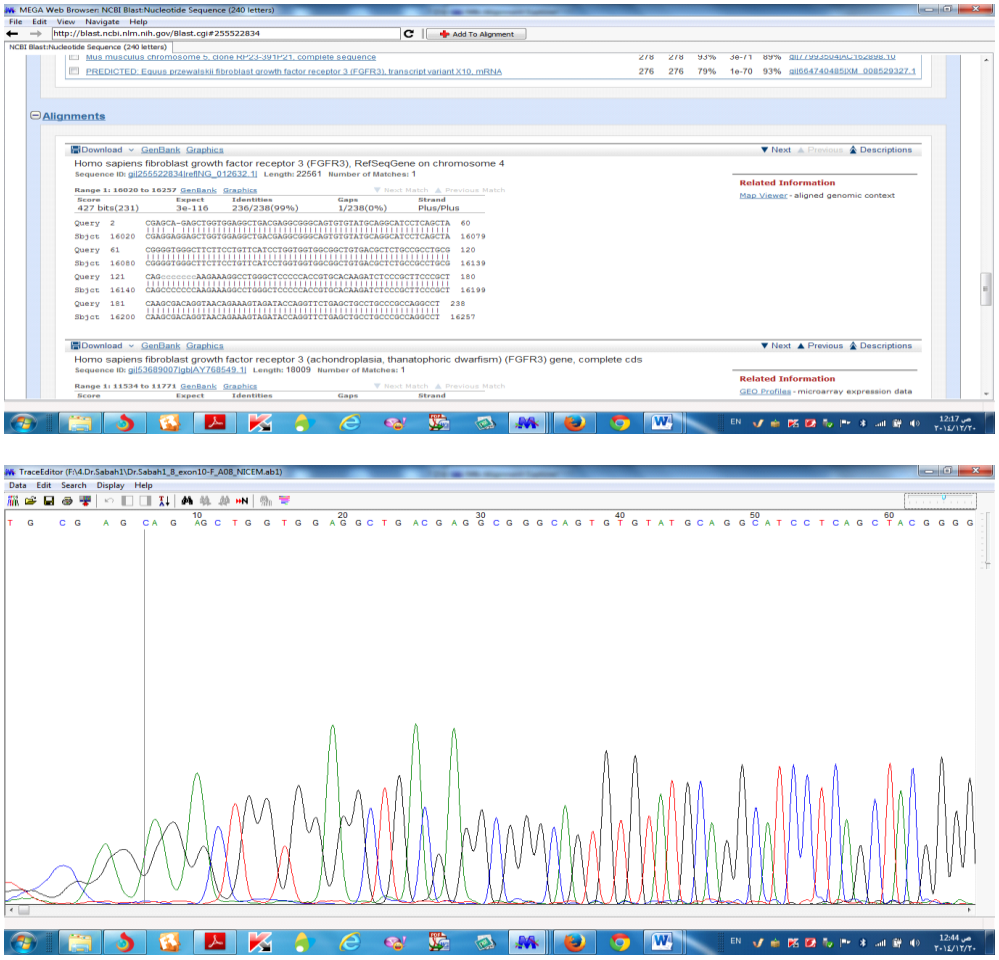

Figure (3): Site 16024 Sub G to C, nucleotide sequence (forward) in exon 9

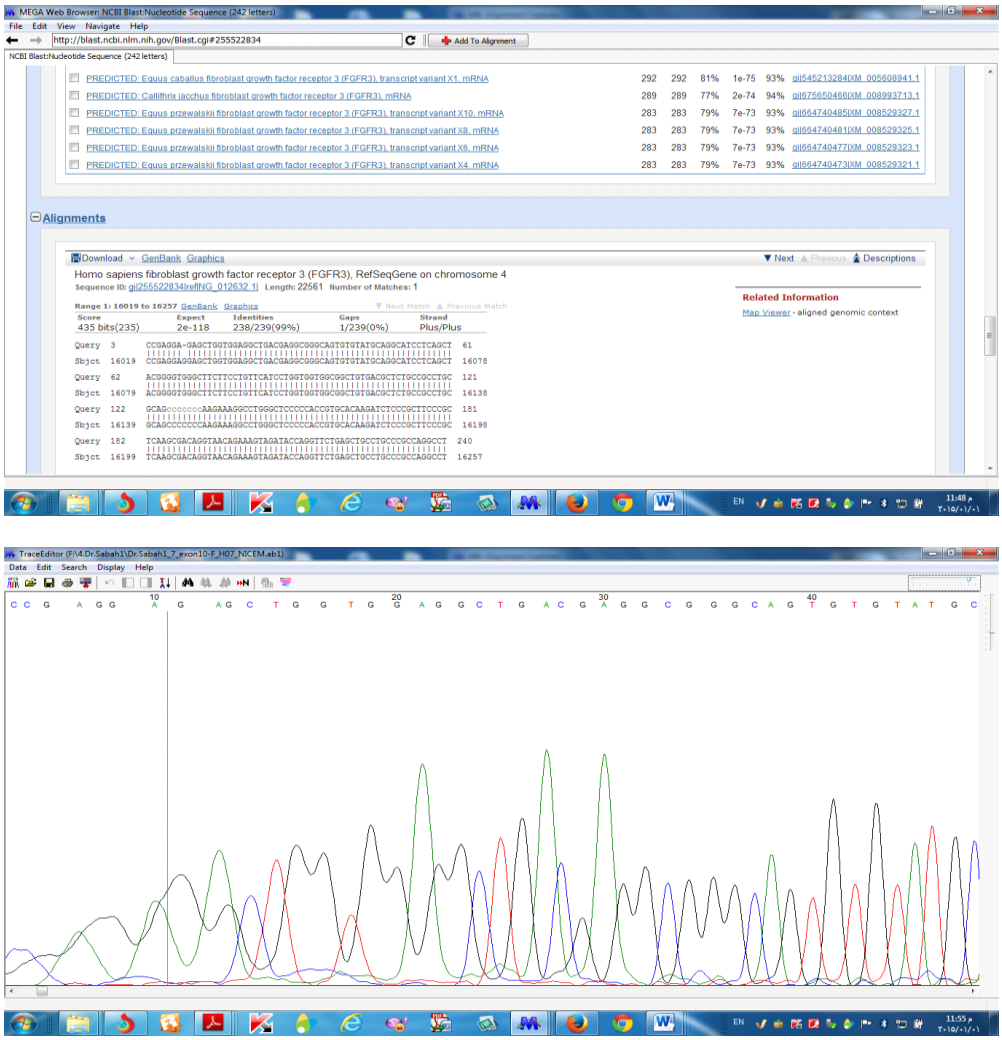

Figure (4): Site 16026 del G, nucleotide sequence (forward) in exon 9

The Association between Bladder Cancer Grade and *FGFR3* Mutations

The study showed that there is a good correlation between the development of bladder cancer and *FGFR3* mutations. The results showed that the exon 9 g.16024 sub G to C has important role in cancer initiation and development since they are detected in the early grade (G1) (38(80.9%) patients of 47). Moreover, the exon 9 mutations (g.16024 sub G to C and g.16026 del G) showed an association with cancer initiation and metastasis since they detected in grand I and II (Tables 3).

Table (3): The number of mutations of the FGFR3 Exon 9 in different cancer grades

Grade	Mutation		Total
	g. 16024 sub G to C (%)	g. 16026 del G (%)	
G1	4 (50.00)	11 (50.00)	15
G2	4 (50.00)	0 (0.00)	4
G3	0 (0.00)	11 (50.00)	11
Total	8	22	30
Chi-square	9.750 **	9.75 **	---
			** (P<0.01).

The results also showed that both g.16024 and g.16026 mutations are needed for G1. While g.16024 and g.16026 mutations are necessary for transforming G1 to G2 and G2 to G3 respectively.

Previous studies indicated a strong correlation between FGFR3 mutations and the stage/grade of the tumor (18, 21). In a study by Khaldon *et al.*, (2010) (30) find a significant correlation ($p < 0.001$) between the stage of the tumor and FGFR3 mutations. However, in contrast to other study, they could not find any correlation between the grade of the tumor and FGFR3 mutations (31). 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 (32, 33). Some of the studies have reported that low-stage and low-grade bladder tumors are associated with *FGFR3* mutations (33, 34). 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 (35, 36). *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) (37). Other studies revealed that no mutations of the *FGFR3* gene in bladder tumor were reported (38, 39).

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