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Molecular and Immunohistochemical Assessment of Fibroblast Growth Factor Receptor 3 Gene as a Survival Time Predictor in Bladder Cancer Patients

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Abstract

BACKGROUND: Mutation signatures contributing to the tumorigenesis of bladder cancer (BC) are complex and heterogeneous, resulting in unpredictable progression, recurrence, and time survival. Clinically, useful prognostic and predictive biomarkers for both disease recurrence and surveillance are therefore needed. Activating fibroblast growth factor receptor 3 (FGFR3) mutations are regarded as early drivers in the molecular pathogenesis of BC.

AIM: The aim of the present study is to evaluate the frequency and distribution pattern of FGFR3 mutation in urine sediments of BC patients in relation to its immunohistochemical (IHC) and molecular expression and to determine the prognostic and predictive value of FGFR3 relative to BC.

PATIENTS AND METHODS: One hundred and sixty patients with diagnosed BC and 80 healthy controls were recruited. Urine samples were collected from all participants. DNA was extracted and FGFR3 mutations were examined in exons 7, 10, and 15 by polymerase chain reaction. IHC for FGFR3 expression and fluorescence *in situ* hybridization technique for assessment of gene amplification was also applied in tissue sections.

RESULTS: Ninety-eight (61.3%) patients were mutant in exon 7, 82 (51.3%) were mutant in exon 10, while only 14 (8.8%) were mutant in exon 15. Univariate logistic regression analysis revealed that mutations in the three exons of FGFR3 were statistically associated with BC and could be used as predictor and/or prognostic parameters for BC. Receiver operating characteristic analysis showed that the mutation of exons 7 and 10 could be used as diagnostic biomarkers for BC. Our findings confirm that FGFR3 mutations are associated with tumors of low grade and stage. The prevalence of mutations was significantly associated with recurrence and survival time of patients for all exons. Kaplan–Meier analysis revealed a significant association between mutant patients in exon 10 and survival time. Our findings suggest that estimation of FGFR3 expression and gene amplification could serve as a prognostic indicator in the follow-up of BC patients. It could also be utilized for molecular targeted therapy in BC.

CONCLUSION: Our data confirmed the feasibility of FGFR3 mutation detection in urine sediment. FGFR3 genetic mutations are independent prognostic factors for tumor recurrence and the genetic alternation of FGFR3 could be used for prediction of survival time of BC patients.

Introduction

Bladder cancer (BC) is a common malignant tumor of the urinary system, with an incidence ranking the first in this system [1]. It has the highest recurrence rate of any malignancy. The most common type is transitional cell carcinoma. It is the 5th most common cancer. It begins when cells in the inner lining of the bladder become abnormal, which causes them to grow and divide out of control. In Egypt, it is ranked the third most common type of cancer in 2020 as mentioned by [2].

Hitherto, the latest shown statistics regarding WHO in Egypt shows that BC comprise about 10, 655 (7.9%) in both sexes.

The National Cancer Institute reported cancer incidence and mortality statistics, for 2022, the estimated number of new cases and deaths are 81,180 and 17,100, respectively [3]. Several factors have been identified that can significantly increase the risk of developing BC such as smoking, alcohols, bilharziasis, and genetic mutations. The limitations of urine cytology and invasiveness of cystoscopy have led to studies searching for the new BC markers that allow for non-invasive detection of bladder carcinomas [4]. The development of reliable and affordable tools to detect BC is a challenge. Genetic and epigenetic alterations in DNA have been reported in the development and progression of BC [5].

Genetic analysis of BC has identified genetic abnormalities in a number of chromosomes and genes that have been known to play a significant role in development of BC pathogenesis and have emerged as a marker for non-aggressive disease and a promising therapeutic target [6]. In the mutational path,

the fibroblast growth factor receptor gene 3 (FGFR3) appears to be the most frequently mutated gene in BC [5].

FGFR3 belongs to the tyrosine kinase family, located at chromosome 4p16.3 and is composed of 19 exons; it plays a significant role in the activation of pathways that controls various cellular functions, such as proliferation, migration, and differentiation [7].

Molecular alteration of FGFR3 gene represents the most recurrent genetic aberrations in BC. Most of the FGFR3 gene mutations occur at 3 hotspots in exons 7, 10, and 15. The presence of FGFR3 mutation in these exons has been evaluated as a marker for recurrence, progression of BC [8].

The goal of the present study is to explore the frequency and distribution pattern of FGFR3 mutation in urine sediments of BC patients in relation to its immunohistological and molecular expression and to validate the prognostic relevance of the incidence of FGFR3 mutations to allow for a future implementation into the diagnostics and therapy in a daily clinical routine.

Subjects and Methods

Patients and samples

A total of 160 patients and 80 healthy volunteers as controls were enrolled in this study, it included a patient criterion of diagnosed BC patients who did not receive any type of therapy, and the diagnosis was confirmed by histopathological examination of the removed tumor tissues by two independent pathologists. Before recruitment, a signed informed consent was obtained from all participants. The research protocol was conducted according to the guidelines of the ethical principles outlined in the declaration of Helsinki and was approved by the Institutional Review Board of the Ethics Committee of Theodor Bilharz Research Institute, in accordance with the institutional guidelines.

Urine samples (100 milliliters) were collected from all patients and controls from the first miction in the morning into a clean sterile container, pooled, and stored at 4°C for up to 72 h prior analysis and were filtered through a single use syringe-filter and the pellet was re-suspended in ×1 Pbs (PH7.2) and stored at −80°C until the DNA extraction.

DNA extraction from urine samples

DNA extraction was carried out using Qiagen DNeasy kit (Hilden, Germany) as per manufacturer instructions, the purified DNA was dissolved in 50 μl of water, measured on a Nanodrop ND-2000c (Thermo Scientific, Waltham, MA, USA), and stored at −20°C for further analysis.

FGFR3 mutation analysis for exons 7, 10, and 15 by polymerase chain reaction (PCR)

Mutation analysis of FGFR3exons 7, 10, and 15 was performed using conventional PCR in a final volume of 25 μl containing 100 ng of urine sediment DNA and the primers used for FGFR3amplification are shown in (Table 1) [9].

Table 1: Primer sequences used and their fragment sizes

Exon	Primer sequence	Fragment size
FGFR3	F- AGT GGC GGT GGTGGT GAG GGA	116 bp
7	R- CAG CAC CGC CGT CTG GTT GG	
10	F- CAA CGC CCA TGT CTT TGC AG	165 bp
	R- GAG CCC AGG CCT TTC TTG G	
15	F- AGG ACA ACG TGA TGA AGA TCG	154 bp
	R- GTG TGG GAA GGC GGT GTT G	

The reaction was conducted at Bio-RAD T100 Thermal cycler as follows: for exon 7 region of FGFR3, initial denaturation 5 min at 95°C, followed by 40 cycle each of denaturation 1 min at 95°C, annealing 1 min at 67°C, extension 1 min at 72°C, and a final extension step at 72°C for 10 min. For exon 10, the same conditions were used except that the annealing was at 64°C and at 62°C for exon 15. Positive controls (DNA of healthy volunteers) were included in each PCR reaction.

PCR products were resolved on 3% agarose gel, electrophoresed on a Bio-RAD electrophoresis chamber, with 5 μl of 100–1000 bp DNA ladder RTU used as a marker and visualized by ethidium bromide staining. The gel image was analyzed using Cleaver micro DOC gel documentation system.

Histopathological examination

Urinary bladder biopsies from the studied groups were fixed in 10% formalin for 24 h, washed in water for 2 h, dehydrated in ascending grades of alcohols, and cleared in xylene. Impregnation was done in pure soft paraffin for 2 h at 55°C, then embedded in hard paraffin blocks. Sections of 5 μm thickness were cut by microtome, stained with hematoxylin, and eosin stain. Bladder tissue sections were blindly-assessed by two pathologists; a screener and a consultant. Sections were examined using light microscope (Scope A1, Axio, Zeiss, Germany) for nature of bladder lesion; benign versus (Vs) malignant, type of malignancy, grade, stage, associated schistosomiasis and carcinoma *in situ* (CIS). Photomicrographs were taken using a microscope-camera (AxioCam, MRc5, Zeiss, Germany).

FGFR3 Immunohistochemistry

The immunohistochemical (IHC) staining procedure was cautiously conducted using the two-step method. Sections were deparaffinized and rehydrated

first, and the slides were heated in PT link (DAKO) for antigen retrieval. Antibody against FGFR3 (FGFR3 B9, Santa Cruz, CA, USA) was diluted according to the manufacture instructions and applied to tissue sections after endogenous peroxidase blocking. Detection Kit (Envision Flex, DAKO) was used. A biotinylated secondary antibody and 3,3-diaminobenzidine (DAB) were applied to tissue sections successively. Slides were counterstained with hematoxylin, dehydrated, and mounted. Positive and negative control sections were stained simultaneously. A semi-quantitative scoring system was adopted, (0) all tumor cells were negative, (1) faint but detectable positivity in some or all cells, (2) weak but extensive positivity, and (3) strong positivity (regardless of extent). In addition to negative controls, sections of ureter and a tumor with known high-level expression were included in each run to represent normal findings (score 1) and strong positivity (score 3), respectively. These acted as reference sections for the scoring of tumors [10].

Fluorescence in situ hybridization (FISH) technique

The probe contains green-labeled polynucleotides (Zygreen) which target FGFR3 gene and orange-labeled polynucleotides which target sequences of chromosome 4 in the chromosomal region 4p11. The code number of the used probe is (ZytoLight® SPEC FGFR3/4p11 Dual Color Probe, Previously: ZytoLightSPECFGFR3/CEN4DualColor Probe).

Sections were de-waxed, incubated for 10 min on a hot plate, followed by incubation twice in xylene, each for 10 min then rehydrated in 100%, 90%, and 70% ethanol, each for 5 min, and lastly washed twice in distilled water, each for 10 min. Sections were incubated for 15 min in pre-warmed pretreatment solution at 98°C, then transferred immediately to distilled water, washed twice, each for 2 min and water was drained off. Pepsin solution was applied to tissue sections that were incubated for 10 min at 37°C in a humid chamber. Then, sections were washed for 5 min in wash buffer (SSC) for 1 min in distilled water. The following steps were, then, successively done: Dehydrationin70%, 90%, and 100% ethanol, each for 1 min. Sections were air dried and treated by 10 μl Zytolight FISH probe onto individual samples. The sections were covered with a coverslip (with hot glue from an adhesive pistol). Denaturation of slides at 75°C for 10 min was done on a hot plate. Sections were then transferred to a humid chamber and hybridized overnight at 37°C. The coverslip was then removed carefully by submerging in ×1 wash buffer twice for 5 min each at 37°C. Dehydration of slides was done in70%, 90%, and 100% each for 1 min.

Cell nuclei were stained by pipetting 30 μl DAP/ Dura-Tect – solution onto the sections with avoiding trapped bubbles. The samples were covered with a coverslip. Excess DAP/Dura-1-solution was carefully removed by gentle pressing the slide between filter papers. The slides were stored in the dark at 2–8°C for longer storage periods.

Interpretation of FISH

Evaluation of the FGFR3 gene was carried out by fluorescence microscopy (Olympus ×51) (using three filters red (FITC), green, and DAPI) and ×1000 oil immersion objective lens. The microscope was attached to a high-resolution video camera (Jale) and monitor. Capturing and interpretation of photos were done using hardware (Cytovision 2.3, USA).

In a normal interphase nucleus, two orange and two green signals are expected. Cells with amplification of FGFR3 gene locus or aneusomy of chromosome 4 will show multiple copies of the green signal or large green signal clusters [11].

Statistical analysis

The data were analyzed using Microsoft Excel 2016 and Statistical Package for the Social Sciences "IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA)." Continuous normally distributed variables were represented as mean ± SD with 95% confidence interval, while non-normal variables were summarized as median with 25 and 75 percentile, and using the frequencies and percentage for categorical variables; $p < 0.05$ was considered statistically significant. To compare the means of normally distributed variables between groups, the Student's t-test was performed, and Mann–Whitney U-test was used in non-normal variables. Chi-square (χ^2) test or Fisher's exact test were used to determine the distribution of categorical variables between groups. Logistic regression analysis was performed to identify predictor associated with the risk of BC occurrence. The Kaplan–Meier method was used to estimate the distribution of survival time for each FGFR3 exon. The diagnostic performance of the studied exons was assessed by receiver operating characteristic (ROC) curves. The area under the ROC (AUC) was calculated as an accuracy index for prognostic performance of selected tests.

Results

Clinical characteristics of BC cases

All the 160 patients who had newly diagnosed BC were admitted for transurethral resection or radical cystectomy. None of the patients had received treatment before the analysis. The demographic and

clinicopathological findings of all cases are depicted in (Table 2).

The mean age of patients at time of diagnosis was 64.1 ± 7.5. They including 127 (79.4%) males and 33 (20.6%) females. Eighty healthy individuals with no history of bladder disease were included as a control group with 37 male (46.3%) and 43 female (53.8%) with mean age of (46.6 ± 12.1). The bladder carcinoma was more prevalent in males in comparison to females and was predominantly seen in older patients.

It is important to highlight that histopathological examinations of the tumors revealed that the greater number was of SqCC 86 (53.75%) than TCC 74 (46.25%), the majority of the tumors were with GII 79 (49.4%), T2 tumor stage 103 (64.4%), single number of tumor mass 87 (54.4%), positive lymph node 83 (51.9%), negative papillary tumor type 126 (78.8%), negative urine cytology 112 (70.0%), negative CIS 129 (80.6%), tumor invasiveness 136 (85.0%), and patients with progressed tumors 136 (85.0%). Notably, most of the tumors demonstrated no tumor recurrence 95 (59.4%) with predominant alive 133 (83.1%) versus died patients.

FGFR3 IHC expression

Both TCC and SqCC showed significantly higher number of positive cases and higher percentage of positive cells compared to the control (Figure 1a). Furthermore, TCC showed significantly higher number of positive cases and non-significantly higher number of positive cells compared to SqCC.

Lower grades of BC showed higher percentage of positive cases for FGFR3 expression, and higher percentage of positive cells compared to higher tumor grade.

Papillary and non-papillary BCs showed non-significant difference considering the percentage of positive cases for FGFR3 expression; however, papillary BC showed significantly higher number of positive cells compared to non-papillary cancer (Table 2, Figure 1c and d).

Similarly, lower stages of BC showed generally higher percentage of cases positive for FGFR3 expression, and higher percentage of positive cells compared to high stages BC.

Table 2: Characteristics of the studied cases and their FGFR3 expression

Parameter	Cases n=160 (%)	Cases positive for FGFR3 immunoexpression (%) Number (%)	Percentage of positive cells (Mean±SD)
Age	64.1±7.5		
Sex			
Female	33 (20.6)		
Male	127 (79.4)		
Schistosomiasis			
Negative	30 (18.8)	24 (80)	47.88±35.12
Positive	130 (81.3)	72 (55.38)[ll]	38.9±28.05
Pathological diagnosis			
Control	80 (100)	22 (27.5)	17.94±7.86
SqCC	86 (53.75)	43 (50)**	39.50±28.11**
TCC	74 (46.25)	53 (71.6)**[$]	44.61±29.78**
Papillary			
Negative	126 (78.8)	77 (61.11)	39.12±25.88
Positive	34 (21.3)	22 (64.71)	53.22±30.15[^^]
Number			
Negative	9 (5.6)		
Single	87 (54.4)		
Multi	64 (40.0)		
Lymph Node			
Negative	77 (48.1)		
Positive	83 (51.9)		
Grade			
GI	19 (11.9)	18 (94.74)	56.51±24.15
GII	79 (49.4)	41 (51.90)	48.47±32.79
GIII	62 (38.8)	22 (35.48)	30.45±28.33
Stage			
T1	30 (18.8)	23 (76.67)	53.11±28.31
T2	103 (64.4)	46 (44.66)	48.22±23.06
T3	24 (15.0)	11 (45.83)	31.44±25.81
T4	3 (1.9)	1 (33.33)	21.07±27.55
Cytology			
Negative	112 (70.0)		
Positive	48 (30.0)		
CIS			
Negative	129 (80.6)		
Positive	31 (19.4)		
Invasiveness			
NMIBC	30 (18.75)	27 (90.0)	52.09±26.15
MIBC	130 (81.25)	62 (47.69)[##]	21.81±27.66[##]
Tumor Progression			
No	24 (15.0)	19 (79.17)	52.11±23.87
Yes	136 (85.0)	62 (45.59)[@@]	25.09±12.44[@@]
Follow-up			
No	95 (59.4)		
RE	65 (40.6)		
Survival			
Live	133 (83.1)		
Die	27 (16.9)		
Survival time	31.4±8.5		

SqCC: Squamous cell carcinoma, TCC: Transitional cell carcinoma, LN: Lymph node, CIS: Carcinoma *in situ*, RE: Recurrence rate, Age and survival time are represented as Mean±SD; the data were analyzed by student t-test. Size is represented as median with interquartile range (25–75%), while the remaining parameters are represented as F (%) frequency and percent; the data were analyzed by X^2 test. **p < 0.01 is highly significant difference with the control group, [ll]High significant difference with the negative group p < 0.01. [$]Significant difference with SqCC p < 0.05. [##]High significant difference with NMIBC p < 0.01. [@@]High significant difference with non-progressive tumors p < 0.01.

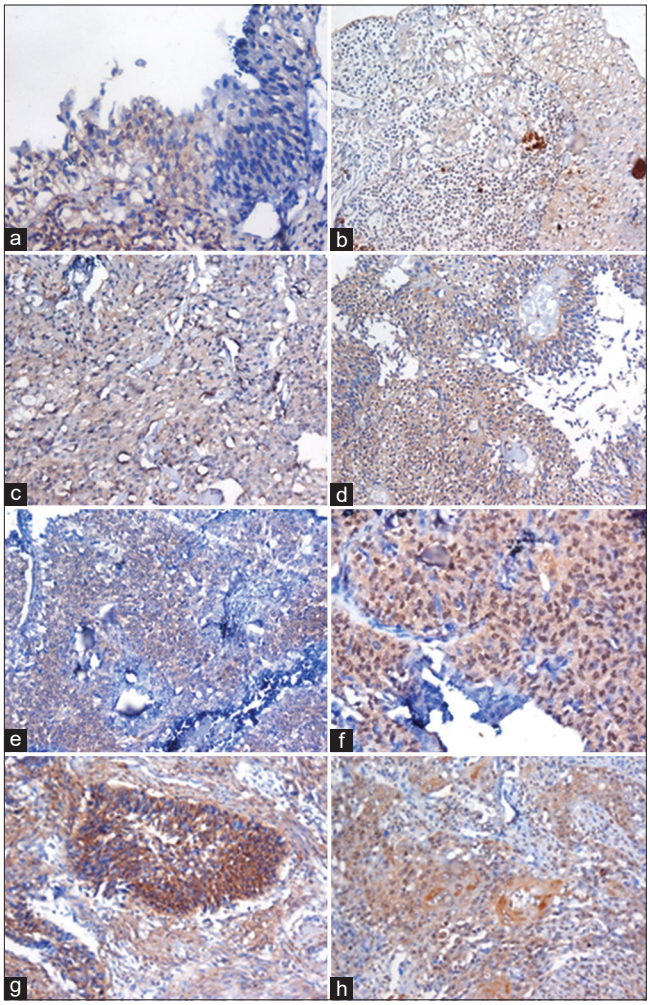

Figure 1: (a) section in bladder wall of cystitis showing mild cytoplasmic expression of FGFR3 in urothelial cells (IHC for FGFR3, DAB, ×400). (b) Section in TCC (G1T1) associated with schistosomiasis showing mild diffuse expression of FGFR3 (IHC for FGFR3, DAB, ×200). (c) Section in bladder wall of non-papillary TCC (G2T1) showing moderate diffuse cytoplasmic expression of FGFR3 in of urothelial cells (IHC for FGFR3, DAB, ×400). (d) Section in papillary TCC (G2T1) showing moderate patchy expression of FGFR3 (IHC for FGFR3, DAB, ×200). (e) Invasive TCC (G2T2), showing marked diffuse nucleocytoplasmic positive expression for FGFR3 (IHC for FGFR3, DAB, ×200). 2 (low power); (f) (high power); and (g) section in invasive, moderately differentiated TCC showing high expression of FGFR3. (h) Invasive SqCC showing high expression of FGFR3 (IHC for FGFR3, DAB, ×400)

Non-invasive BC showed higher percentage of positive cases with expression of FGFR3 and higher percentage of positive cells compared to invasive BC (Figure 1e and f, Table 2).

Progressive BCs showed lower percentage of positive cases expressing FGFR3 and lower percentage of positive cells compared to non-progressive BC (Table 2).

Schistosomiasis-associated BC showed significantly less number of positive cases for FGFR3 expression (Figure 1b) and non-significantly less percentage of positive cells than schistosomiasis-non-associated (Table 2, Figure 1g and h).

Assessment of FGFR3 gene amplification by FISH

FISH technique was applied to60 malignant cases and 20 benign cases exhibiting overexpression of the FGFR3 protein by immunohistochemistry (Table 3).

Table 3: FGFR3 gene amplification in studied groups

Group	Positive FGFR3 Gene Amplification Number (%)	Negative FGFR3 Gene Amplification Number (%)	p-value
Benign cases (20)	0 (0)	20 (100)	<0.01
Malignant cases (60)	54 (90)	6 (10)	<0.01
Low grade (28)	25 (89.29)	3 (10.71)	<0.01
High grade (32)	29 (90.06)	3 (10.34)	
NMIBC (32)	27 (84.38)	5 (15.62)	<0.01
MIBC (28)	27 (96.44)	1 (3.56)	
TCC (34)	30 (88.24)	4 (11.76)	<0.01
Low grade (23)	20 (86.96)	3 (13.04)	<0.01
High grade (11)	10 (90.91)	1 (9.09)	
NMIBC (27)	24 (88.89)	3 (11.11)	<0.01
MIBC (7)	6 (85.71)	1 (14.29)	
Schistosoma associated (7)	7 (100)	0 (0)	<0.01
Schistosoma non-associated (27)	23 (85.19)	4 (14.81)	
SqCC (26)	23 (88.46)	3 (11.54)	<0.01
Low grade (5)	5 (100)	0 (0)	<0.01
High grade (21)	18 (85.71)	3 (14.29)	
NMIBC (5)	4 (80)	1 (20)	<0.01
MIBC (21)	19 (90.48)	2 (9.52)	
Schistosoma associated (26)	23 (88.46)	3 (11.54)	<0.01
Schistosoma non-associated (0)	0 (0)	0 (0)	

Fifty-four malignant cases out of 60 showed amplified FGFR3 gene. Positive malignant cases showed a significant difference compared to negative cases at $p < 0.01$.

Most of low- and high-grade malignant cases (89.29% and 90.06%, respectively) showed positive gene amplification. Furthermore, both non-muscle invasive BC (NMIBC) and muscle-invasive BC (MIBC) showed high percentage of positive gene amplification (84.38% and 96.44%, respectively).

A total of 88.24% of TCC cases showed FGFR3 gene amplification, with most of low-grade (86.96%) and high-grade tumors (90.91%) showed positive gene amplification, with no significant difference at $p > 0.05$ of low-grade compared to high-grade ones. (Figure 2a and b).

In NMIBC group, 88.89% of cases showed positive gene amplification, while MIBC cases showed positive gene amplification in (85.71%) with no statistical difference.

Most cases of SqCC have FGFR3 gene amplification (88.46%) with most of the positive cases which were of high grade and belong to the MIBC group (90.48%) with a significant difference at $p < 0.01$ compared to NMIBC. Most cases of SqCC (88.46%) and all TCC associated with schistosomiasis report FGFR3 gene amplification (Table 3, Figure 2c and d).

FGFR3 mutation analysis

Mutations of FGFR3gene were analyzed in 160 DNAs extracted from urine sediments of BC patients, the statistical analysis allowed for the detection of

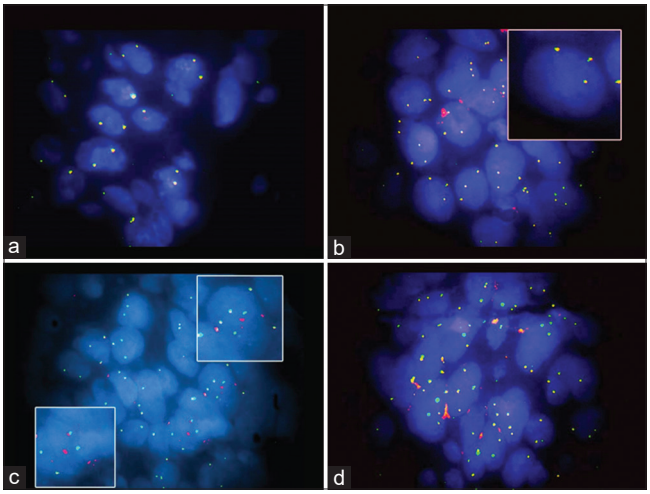

Figure 2: (a) TCC (Ta GI) showing no amplification of FGFR3 as two FGFR3 signals (green) and two chromosome 4 signal (orange/red) (FISH, magnification ×1000), (b) low grade, T1, TCC, SPEC FGFR3 Dual Color Probe hybridized with interphase cells showing abnormal chromosome 4 as indicated by multiple green and orange signals in the nuclei (magnification ×1000), (c) urinary bladder tissue from a case of TCC T1 GII; SPEC FGFR3 Dual Color probehybridized with interphase cells showing polysomy of chromosome 4 as indicated by multiple green and orange signals in the nuclei (magnification ×1000), (d) urinary bladder tissue from a case of SqCC; SPEC FGFR3 Dual Color Probe hybridized with interphase cells showing an amplification of the FGFR3 gene (green signals) (magnification ×1000)

98 patients (61.3%) mutant in exon 7, 82 (51.3%) mutant in exon 10, while only 14 (8.8%) mutant in exon 15 (Table 4).

Table 4: The frequency of the studied exons and the viability to use as prognostic biomarkers

Exons of FGFR	Groups			Prognostic viability	
	Control n = 80 (%)	Cases n = 160 (%)	p-value	OR (95% C.I)	p-value
Exon 7					
Wild	80 (100.0)	62 (38.8)	0.001**	0.44 (0.36–0.53)	0.001**
Mutant	0 (0.0)	98 (61.3)			
Exon 10					
Wild	80 (100.0)	78 (48.8)	0.001**	0.49 (0.42–0.58)	0.001**
Mutant	0 (0.0)	82 (51.3)			
Exon 15					
Wild	80 (100.0)	146 (91.3)	0.003	0.65 (0.59–0.71)	0.001**
Mutant	0 (0.0)	14 (8.8)			

The distribution of the studied exons is represented as F (%) frequency and percent; the data were analyzed by X^2 test. OR: Odd ratio, C.I: Confidence interval, p-value of prognostic viability is calculated depending on logistic regression analysis. *p < 0.05 is significant, **p < 0.01 is highly significant.

Univariate logistic regression analysis was performed for relevant indexes to identify potential prognostic risk factors for BC. It was carried out for the 3 exons mutation to evaluate their viability to use as prognostic biomarkers, the analysis showed that all of the studied exons were statistically associated with BC, the mutation of exons 7, 10, and 15 of FGFR3 gene may be used as predictor and/or prognostic parameters for BC prospection, an increase in 1 degree of exon 7, 10, and 15, increased the odds of being BC by a factor of odd ratio and C.I; confidence interval OR (95% C.I) = 0.44 (0.36–0.53), 0.49 (0.42–0.58) and 0.65 (0.59–0.71), respectively, with (p = 0.001) (Table 4).

Association of FGFR3 mutations with patients outcome

Evaluation of the relationship between presence of FGFR3 mutations and patient gender/age revealed that there was no significant association between the occurrence of FGFR3 mutations and patient's age or gender, while mutations of exon 15 were significantly associated with gender.

For exon 7, an association was observed between the occurrence of mutation with squamous cell carcinoma patients with OR (95% C.I) = 2.83 (1.25–3.48) (p = 0.001), patients with papillary tumor OR (95% C.I) = 2.42 (1.19–03.91) (p = 0.03), with larger tumor size OR (95% C.I) = 1.14 (1.09–1.20) (p = 0.001), patients with GII OR (95% C.I) = 12.0 (5.22–27.60) (p = 0.001), patients with T2 stage OR (95% C.I) = 1.91 (1.25–2.91) (p = 0.05), patients with positive CIS OR (95% C.I) = 7.36 (2.12–25.59) (p = 0.001), with tumor recurrence OR (95% C.I) = 0.3 (0.3–0.5) (p = 0.001), and, finally, an association was also observed between the mutation occurrence and survival time of patients with OR (95% C.I) = 0.5 (0.5–0.6) (p = 0.001) and mean survival time of 31.4 ± 8.5 (Table 5).

Regarding exon 10, a significant association was found with sex as 70 (85.4%) males were mutant with OR (95% C.I) = 2.1 (1.0–4.7) (p = 0.06). The clinicopathological factors which were significantly associated with the overall occurrence of mutation included, squamous cell carcinoma patients OR (95% C.I) = 1.8 (0.89–2.36) (p = 0.03), patients with multi number of tumor mass OR (95% C.I) = 62.43 (17.72–219.99) (p = 0.001), patients with larger tumor size OR (95% C.I) = 1.09 (1.06–1.13) (p = 0.001), patients with GII OR (95% C.I) = 2.71 (1.65–4.48) (p = 0.001), patients with positive CIS OR (95% C.I) = 4.89 (1.86–12.82) (p = 0.001), with tumor recurrence OR (95% C.I) = 0.2 (0.1–0.3) (p = 0.001), survival time of patients with OR (95% C.I) = 10.3 (3.0–36.0) (p = 0.001), and mean survival time of 32.4 ± 8.6. Finally, the results revealed that the presence of mutation in exon 10 may extend the life time of the patient and the observed significant difference was between the wild and mutant patients with p = 0.01 and this significance does not lead to BC risk (Table 5).

While regarding exon 15, the results showed an association between mutation occurrence with sex with OR (95% C.I) = 1.11 (1.05–1.18) (p = 0.05) and what catches our attention that all the mutant patients were only males. The clinicopathological factors which were significantly associated with mutation occurrence included, squamous cell carcinoma patients OR (95% C.I) = 2.86(0.79–3.94) (p = 0.001), patients with multi number of tumor mass OR (95% C.I) = 1.25 (1.10–1.42) (p = 0.001), patients with larger tumor size OR (95% C.I) = 1.05 (1.00–1.10) (p = 0.04), patients with T1 stage OR (95% C.I) = 2.29 (1.12–3.71) (p = 0.01),

Table 5: Association between FGFR3mutation status and clinicopathological features in BC

Parameter	Exon7					Exon10					Exon15				
	Wild n = 62 (%)	Mutant n = 98 (%)	p-value	Risk assessment		Wild n = 78 (%)	Mutant n = 82 (%)	p-value	Risk assessment		Wild n = 146 (%)	Mutant n = 14 (%)	p-value	Risk assessment	
				OR (95% C.I)	p-value				OR (95% C.I)	p-value				OR (95% C.I)	p-value
Age	63.8 ± 7.3	64.4 ± 7.3	0.6	1.01 (0.97–1.06)	0.6	63.5 ± 8.2	64.8 ± 6.3	0.3	1.02 (0.98–1.07)	0.3	64.5 ± 6.7	60.3 ± 12.2	0.3	0.93 (0.86–1.00)	0.5
Sex															
Female	9 (14.5)	24 (24.5)	0.1	0.54 (0.23–1.26)	0.2	21 (26.9)	12 (14.6)	0.04*	2.1 (1.0–4.7)	0.05*	33 (22.6)	0 (0.0)	0.04*	1.11 (1.05–1.18)	0.05*
Male	53 (85.5)	74 (75.5)				57 (73.1)	70 (85.4)				113 (77.4)	14 (100.0)			
Pathological diagnosis															
SqCC	15 (24.2)	71 (72.4)	0.001**	2.83 (1.25–3.48)	0.0011	35 (44.9)	54 (65.9)	0.02*	1.8 (0.89–2.36)	0.03*	75 (51.4)	14 (100.0)	0.001**	2.86 (0.79–3.94)	0.001**
TCC	47 (75.8)	24 (24.5)	0.001**	0.12 (0.06–0.26)	0.001**	43 (55.1)	28 (34.1)	0.01*	0.45 (0.23–0.86)	0.02*	71 (48.6)	0 (0.0)	-	-	-
Papillary															
Negative	0 (0.0)	3 (3.1)	0.02*	2.42 (1.19–03.91)	0.03*	56 (71.8)	70 (85.4)	0.04*	0.44 (0.20–0.98)	0.05*	115 (78.8)	11 (78.6)	0.5	1.20 (0.31–4.71)	0.6
Positive	43 (69.4)	83 (84.7)				22 (28.2)	12 (14.6)				31 (21.2)	3 (21.4)			
Number															
Single	19 (30.6)	15 (15.3)	0.001**	0.37 (0.28–0.48)	0.001**	68 (87.2)	19 (23.2)	0.001**	62.43 (17.72–219.99)	0.001**	87 (59.6)	0 (0.0)	0.001**	1.25 (1.10–1.42)	0.001**
Multi	53 (85.5)	34 (34.7)				4 (5.1)	60 (73.2)				50 (34.2)	14 (100.0)			
Size	2.0 (1.5–4.0)	14.0 (9.9–27.5)	0.001**	1.14 (1.09–1.20)	0.001**	6.8 (1.9–22.0)	20.0 (12.4–29.4)	0.001**	1.09 (1.06–1.13)	0.001**	6.8 (1.9–22.0)	20.0 (12.4–29.4)	0.03*	1.05 (1.00–1.10)	0.04*
Lymph Node															
Negative	19 (30.6)	58 (59.2)	0.001**	0.33 (0.17–0.67)	0.001**	26 (33.3)	51 (62.2)				63 (43.2)	14 (100.0)	0.001**	0.83 (0.75–0.92)	0.001**
Positive	43 (69.4)	40 (40.8)				52 (66.7)	31 (37.8)	0.001**	0.31 (0.16–0.61)	0.001**	83 (56.8)	0 (0.0)			
Grade															
GI	46 (74.2)	16 (16.3)	0.001**	0.29 (0.15–0.54)	0.001**	49 (62.8)	13 (15.9)	0.001**	0.20 (0.10–0.41)	0.001**	62 (42.5)	0 (0.0)	0.1	-	-
GII	7 (11.3)	72 (73.5)	0.001**	12.00 (5.22–27.60)	0.001**	23 (29.5)	56 (68.3)	0.001**	2.71 (1.65–4.48)	0.001**	75 (51.4)	4 (28.6)	0.001**	0.04 (0.01–0.13)	0.001**
GIII	9 (14.5)	10 (10.2)	0.7	1.00 (0.40–2.52)	0.9	6 (7.7)	13 (15.9)	0.1	2.00 (0.75–5.33)	0.2	9 (6.2)	10 (71.4)	0.9	1.00 (0.40–2.52)	0.9
Stage															
T1	9 (14.5)	21 (21.4)	0.1	2.00 (0.90–4.45)	0.2	10 (12.8)	20 (24.4)	0.08	2.00 (0.90–4.45)	0.09	23 (15.8)	7 (50.0)	0.01*	2.29 (1.12–3.71)	0.01*
T2	38 (61.3)	65 (66.3)	0.04*	1.91 (1.25–2.91)	0.05*	50 (64.1)	53 (64.6)	0.4	1.13 (0.76–1.69)	0.5	96 (65.8)	7 (50.0)	0.001**	0.07 (0.03–0.15)	0.001**
T3	15 (24.2)	9 (9.2)	0.01*	0.60 (0.26–1.37)	0.01*	18 (23.1)	6 (7.3)	0.01*	0.33 (0.13–0.84)	0.02*	24 (16.4)	0 (0.0)	0.1	-	-
T4	0 (0.0)	3 (3.1)	-	-	-	0 (0.0)	3 (3.7)	-	-	-	3 (2.1)	0 (0.0)	0.2	-	-
Cytology															
Negative	39 (62.9)	73 (74.5)	0.1	0.61 (0.50–0.75)	0.2	52 (66.7)	60 (73.2)	0.4	0.74 (0.37–1.48)	0.4	105 (71.9)	7 (50.0)	0.1	2.54 (0.77–8.35)	0.2
Positive	23 (37.1)	25 (25.5)				26 (33.3)	22 (26.8)				41 (28.1)	7 (50.0)			
CIS															
Negative	59 (95.2)	70 (71.4)	0.001**	7.36 (2.12–25.59)	0.001**	72 (92.3)	57 (69.5)	0.001**	4.89 (1.86–12.82)	0.001**	115 (78.8)	14 (100.0)	0.06	0.90 (0.85–0.96)	0.07
Positive	3 (4.8)	28 (28.6)				6 (7.7)	25 (30.5)				31 (21.2)	0 (0.0)			
Invasiveness															
Non	9 (14.5)	15 (15.3)	0.9	0.9 (0.4 –2.3)	0.9	10 (12.8)	14 (17.1)		0.7 (0.3–1.7)	0.6	17 (11.6)	7 (50.0)	0.001**	0.1 (0.04–0.4)	0.001**
Invasive	53 (85.5)	83 (84.7)				68 (87.2)	68 (82.9)	0.5			129 (88.4)	7 (50.0)			
Tumor Progression															
No	9 (14.5)	15 (15.3)	0.9	0.9 (0.4–2.3)	0.9	10 (12.8)	14 (17.1)	0.5	0.7 (0.3–1.7)	0.6	17 (11.6)	7 (50.0)	0.001**	0.1 (0.04–0.4)	0.001**
Yes	53 (85.5)	83 (84.7)				68 (87.2)	68 (82.9)				129 (88.4)	7 (50.0)			
Follow-up															
No	62 (100.0)	33 (33.7)	0.001**	0.3 (0.3–0.5)	0.001**	78 (100.0)	17 (20.7)	0.001**	0.2 (0.1–0.3)	0.001**	91 (62.3)	4 (28.6)	0.01*	4.1 (1.2–13.8)	0.01*
RE	0 (0.0)	65 (66.3)				0 (0.0)	65 (79.3)				55 (37.7)	10 (71.4)			
Survival															
Alive	62 (100.0)	71 (72.4)	0.001**	0.5 (0.5–0.6)	0.001**	75 (96.2)	58 (70.7)	0.001**	10.3 (3.0–36.0)	0.001**	125 (85.6)	8 (57.1)	0.01*	4.5 (1.4–14.2)	0.01*
Died	0 (0.0)	27 (27.6)				3 (3.8)	24 (29.3)				21 (14.4)	6 (42.9)			
Survival time	-	31.4 ± 8.5	-	-	-	24.0 ± 0.3	32.4 ± 8.6	0.01*	1.21 (0.91–1.62)	0.2	29.4 ± 8.6	38.5 ± 1.6	0.01*	1.16 (1.01–1.35)	0.03*

Age and survival time are represented as Mean ± SD; the data were analyzed by student t-test. While sex, pathological diagnosis, papillary, number, LN, grade, stage, cytology, CIS, invasiveness, tumor progression, follow-up, and survival are represented as F (%) frequency and percent; the data were analyzed by X^2 test. However, size of tumor is represented as median with interquartile range (25–75%), the data were analyzed by Mann–Whitney U-test. OR: Odd ratio, C.I: Confidence interval, p-value of risk assessment is calculated depending on logistic regression analysis. *p < 0.05 is significant, **p < 0.01 is highly significant.

with tumor recurrence OR (95% C.I) = 4.1 (1.2–13.8) (p = 0.01), and, finally, with survival time of patients with OR (95% C.I) = 4.5 (1.4–14.2) (p = 0.01) and mean survival time of 38.5 ± 1.6 (Table 5).

Statistical evaluation of diagnostic performance of FGFR3 mutation using ROC analysis

Statistical evaluation of diagnostic performance in general and ROC analysis in particular is important for assessing the efficacy of the clinical utility of the studied mutations as a diagnostic biomarker. It may include evaluation of sensitivity, specificity, predictive values, odds ratios, accuracy, and AUC.

ROC curve was established to assess the diagnostic performance of the mutation in exons 7, 10, and 15 of FGFR3 gene in BC patients and to evaluate the specificity and sensitivity of BC prediction.

Our results indicated that mutation of exon 7 was with sensitivity of 61.3%, specificity of 100.0% with an AUC of 0.806 (p < 0.0001, 95% C.I: 0.753–0.859), and accuracy 61.3%. The mutation of exon 10 was with sensitivity of 51.3% and specificity of 100.0% with an AUC of 0.756 (p < 0.0001, 95% C.I: 0.698–0.815) and accuracy 51.3%, while the mutation of exon 15 was with sensitivity of 8.8% and specificity of 100.0% with an AUC of 0.544 (p = 0.269, 95% C.I: 0.469–0.619) and accuracy 8.8%. The above results showed that the mutation of exons 7 and 10 could be used as diagnostic biomarkers for BC (Table 6 and Figure 3).

Table 6: Diagnostic performance

Studied markers	Sn.	Sp.	PPV	NPV	Accuracy	AUC	95%C.I	p-value
FGFR								
Exon7	61.3	100.0	100.0	46.7	61.3	0.806	0.753–0.859	<0.0001
Exon10	51.3	100.0	100.0	41.0	51.3	0.756	0.698–0.815	<0.0001
Exon15	8.8	100.0	100.0	26.6	8.8	0.544	0.469–0.619	0.269

Sn: Sensitivity, Sp: Specificity, PPV: Positive predictive value, NPV: Negative predictive value, AUC: Area under curve and C.I: 95% Confidence Interval. *p < 0.05 is significant, **p < 0.01 is highly significant.

The Kaplan–Meier (KM) method is a popular method to analyze "time-to-event" data. It is very useful in survival analysis as it is used by the researchers to determine and/or analyze the patients or participants who lost to follow-up or dropped out of the study, those who developed the disease of interest or survived it.

In our study, we found that the survival time was much closer in the cases with mutation in exon 7, the mean time to survive in mutants was 31.444 months with 95%C.I. of (28.233–34.656), For mutants of exon 10, a significant association was observed between mutant patients, and survival time with mean time to survive in mutants was 32.375 months with 95%C.I. of (28.938–35.812) and Log Rank (Mantel-Cox)= 4.667 and p = 0.031, while no statistical significant association was found regarding mutant patients in exon 15 as the mean time to survive was 38.500 months with 95%C.I. of (37.185–39.815) and Log Rank (Mantel-Cox) = 1.357 and p = 0.244 (Table 7 and Figure 4).

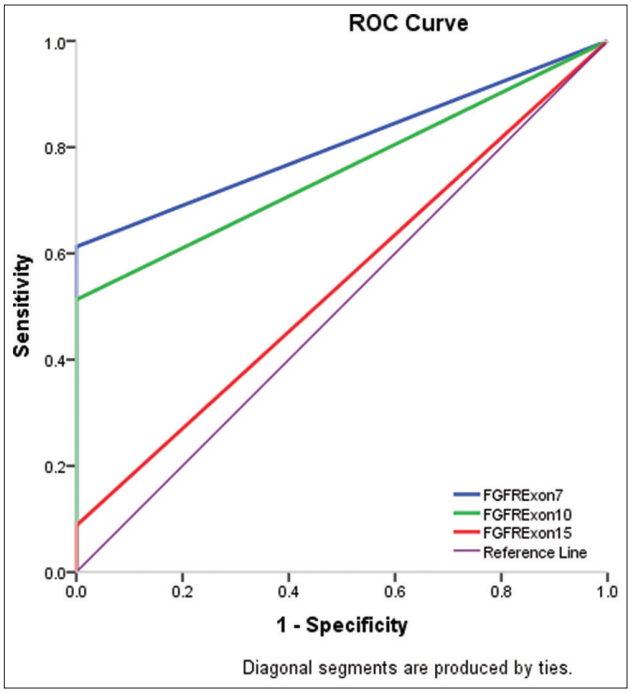

Figure 3: ROC curve of the studied exons of FGFR3 gene

Table 7: Kaplan–Meier analysis of FGFR3 mutation regarding survival time

Parameter	Mean estimate	Std. Error	95% Confidence Interval		Log Rank (Mantel-Cox)	p-value
			Lower bound	Upper bound		
Exon7						
Wild	-	-	-	-	N.A	N.A
Mutant	31.444	1.639	28.233	34.656		
Exon10						
Wild	24.000	0.000	24.000	24.000	4.667	0.031*
Mutant	32.375	1.754	28.938	35.812		
Exon15						
Wild	29.429	1.882	25.740	33.117	1.357	0.244
Mutant	38.500	0.671	37.185	39.815		

p-value depending on the Kaplan–Meier test. *p < 0.05 is significant, **p < 0.01 is highly significant.

Association between tumor grade of FGFR3 mutations and survival time

Using ANOVA test, the survival time of mutant patients was associated to tumor grade and stage, the results revealed the presence of significant association between mutants in exon 7 and GI patients with mean time of 40.2 ± 1.3 months and p = 0.007, this means that mutant patients in exon 7 and diagnosed GI have the chance to survive more time than mutant patients in the same exon and diagnosed GII or GIII, and likewise, a significant association was observed between mutant patients in exon 10 and GI patients with mean time of 38.5 ± 1.6 months and p = 0.04, while for exon 15, a significant association was observed between survival time and mutant patients with GII tumor with mean time 31.9 ± 9.4 months and p = 0.05 as there was no mutants were detected with GI (Table 8).

Table 8: Survival time regarding the associations between the tumor grade and the studied FGFR mutant exons

Mutant	GI	GII	GIII	p-value
Exon 7	40.2 ± 1.3	32.2 ± 7.4	23.5 ± 0.01	0.007*
Exon 10	38.5 ± 1.6	31.6 ± 9.3	24.0 ± 0.01	0.04*
Exon 15	-	31.9 ± 9.4	25.0 ± 0.01	0.05*

Survival time is represented as Mean ± SD; the data were analyzed by ANOVA test. *p < 0.05 is significant, ** p < 0.01 is highly significant.

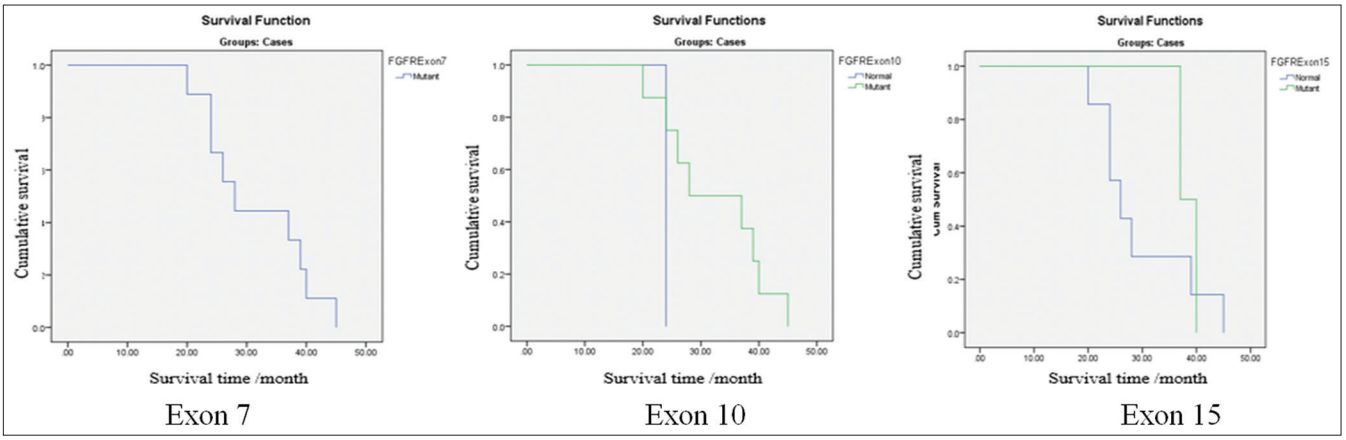

Figure 4: Kaplan–Meier analysis of FGFR3 mutation regarding survival time

Association between tumor stage of FGFR3 mutations and survival time

The association between survival time of mutant patients and tumor stage was performed, the analyzed data revealed the presence of significant association between mutants in exon 7 and T1 patients with mean time of 36.6 ± 0.01 months and p = 0.05, this indicates that mutant patients in exon 7 and staged T1 have the chance to survive more time than mutant patients in the same exon and diagnosed T2, T3, or T4, this means, there is an inverse association between the tumor stage and the survival time.

From these associations, we can confirm that survival time decreases in mutant patients as tumor grade gets higher and advanced stage. No significant association was found between survival time and mutant patients in exon 10 as well as those in exon 15 (Table 9).

Table 9: Survival time regarding the associations between the tumor stage and the studied FGFR mutant exons

Mutant	T1	T2	T3	T4	p-value
Exon 7	36.6 ± 0.01	34.8 ± 8.3	27.7 ± 8.7	24.0 ± 0.02	0.05*
Exon 10	37.0 ± 0.02	34.2 ± 7.9	29.5 ± 10.4	23.9 ± 0.01	0.1
Exon 15	36.5 ± 0.03	40.0 ± 0.01	-	-	0.8

Survival time is represented as Mean ± SD; the data were analyzed by ANOVA test. *p < 0.05 is significant, **p < 0.01 is highly significant.

Discussion

BC is characterized by high incidence and recurrence rates together with genomic instability and elevated mutation degree. At present, cystoscopy combined with cytology is routinely used for diagnosis, prognosis, and disease surveillance. Such an approach is often associated with several side effects, discomfort for the patient, and high economic burden. Thus, there is an essential demand of non-invasive, sensitive, fast, and inexpensive biomarkers for clinical management of BC patients [12].

The present study examined the utility of FGFR3 mutation status as a prognostic marker in the urine sediments of 160 BC patients diagnosed histopathologically on bladder tissue specimens. Here, we chose urine because it is non-invasive way for mutational analysis of FGFR3. It is more specific for BC than any other body fluid, because urine comes into direct contact with bladder tumors and it is easily accessible. Evidence of the success of using urine is the study of [13], in which nine out of 28 patients exhibited FGFR alterations (32%) based on tissue testing, while 8 out of 21 matched urine samples were FGFR positive (38%) and they reported that there were three patients being FGFR positive from urine with no mutation found in the corresponding tissue biopsy.

Both TCC and SqCC showed significantly higher number of positive cases and higher percentage of positive cells compared to the controls. This finding is compatible with [14]. There was no evidence of such staining in normal urothelium. We also found that 60% of the malignant cases were positive for FGFR3 immunostaining, which is compatible with [15] who stated that expression of FGFR3 was found in approximately 70% of both low- and high-grade tumors, as well as equally distributed between invasive and non-invasive urothelial carcinoma.

Furthermore, TCC showed significantly higher number of positive cases and non-significantly higher number of positive cells compared to SqCC cases. About half of the SqCC cases in the present study were positive for FGFR3 immunostaining, and this was also explained by [16] who stated that SqCC showed a predisposition to a high level of FGFR3 protein expression and mutation.

Papillary and non-papillary BCs showed non-significant difference considering the percentage of positive cases for FGFR3 expression; however, papillary BC cases showed significantly higher number of positive cells compared to non-papillary cases.

Lower grades of BC showed higher percentage of positive cases for FGFR3 expression, and higher percentage of positive cells compared to higher tumor grade. About 60% of low-grade urothelial cancers of our cases (GI+GII) were positive for FGFR3

immunostaining, and this is compatible with [17] who found that 69.4% of low-grade urothelial carcinoma was positive to FGFR3, while only 35% of high-grade tumors were positive for FGFR3. Similarly, lower stages of BC showed generally higher percentage of positive cases for FGFR3 expression, and higher percentage of positive cells compared to advanced stages of BC cases.

Non-invasive BC showed higher percentage of positive cases with expression of FGFR3 and higher percentage of positive cells compared to invasive BC.

Progressive BCs showed lower percentage of positive cases expressing FGFR3 and lower percentage of positive cells compared to non-progressive BC. In our study, 90% of non-invasive tumors showed positive expression of FGFR3. Another study was conducted on 255 primary urothelial carcinomas reported that 63.5% of superficial carcinoma were positive for FGFR3 protein expression by immunophenotyping, while only 33% were positive of invasive muscle tumors [17].

Schistosomiasis-associated BC showed significantly less number of positive cases for FGFR3 expression and non-significantly less percentage of positive cells than schistosomiasis-non-associated.

FISH technique was applied to 60 malignant cases and 20 benign cases with overexpression of the FGFR3 protein. Fifty-four malignant cases out of 60 showed amplified FGFR3 gene. Positive malignant cases show a significant difference compared to negative cases at p-value <0.01. Our results are close to [10] who mentioned that an association was found between expression level and mutation status.

Most of low- and high-grade malignant cases showed positive gene amplification. Furthermore, both NMIBC and MIBC cases showed high percentage of positive gene amplification (84.38% and 96.44%), respectively. These results are slightly higher to what was reported by [15] who stated that 63% of NMIBC group and 59% of MIBC group show FGFR3 gene amplification.

In NMIBC group, 88.89% of cases showed positive gene amplification, while MIBC cases showed positive gene amplification in (85.71%) with no statistical difference between NMIBC and MIBC groups. This result was correlated to that of [18] who detected FGFR3 gene amplification in 88.2% of the TCC cases, in which 70% were of low grade, and 80% were in NMIBC group.

Most cases of SqCC have FGFR3 gene amplification (88.46%) with most of the positive cases were of high grade and belong to the MIBC group (90.48%) with a significant difference at $p < 0.01$ compared to high grade and NMIBC group. Similar results were also achieved by [10] who stated that there is an association between expression level and mutation status [19] reported in their study that all amplified samples showed concomitant FGFR3 mutations and protein overexpression. Most cases of SqCC (88.46%) and all TCC associated with schistosomiasis report FGFR3 gene amplification. They were mostly of high grade and were in MIBC group. This was previously mentioned by [20] who explained that by chromosomal alterations that characterize Schistosomal BC cases.

The prevalence and distribution of FGFR3 mutations and their association with outcome in BC patients was reported. The results revealed the presence of 98 patients (61.3%) mutant in exon 7, 82 (51.3%) mutant in exon 10, while only 14 (8.8%) mutant in exon 15. The high frequency of mutations that we found confirmed that FGFR3 mutations are a frequent event in bladder carcinomas and may be due to environmental factors as smoking, exposure to chemicals, and gasses, microbial, and parasitic infections as Schistosomiasis and hepatitis C virus infection (HCV) that could induce several genetic mutations in Egyptian patients. A study for [21] was published with higher FGFR3 mutation frequency than our study; they showed FGFR3mutations in 12 of 13 (92.3%) tumor tissues and 11 of 13 (84.6%) urine samples from patients with superficial BC. The presence of high frequency of FGFR3 mutations confirming its implication in bladder carcinogenesis, as suggested by Pouessel *et al.*, 2018 [22].

Our findings coincide with those of [23] who reported that mutations are clustered in three hotspots in exons 7, 10, and 15 and the most common mutation (up to 70% of tumors harboring FGFR3 mutations) occurs in exon 7, while mutations in other exons are less common.

As reported by [8] who performed the study on Indian patients, that the frequency of FGFR3 mutations varies significantly across different countries, and they explained the difference in frequency is due to geographical region, sample size, and methodology used for mutation detection.

In general, our results demonstrated a higher abundance of mutations in patients with squamous cell carcinoma and notably, in exon 15, all the mutant patients were with sqCC, while patients with TCC were abundant in wild patients in all the three studied exons.

The prognostic viability of the 3 exon mutations was determined and the results showed that they were statistically associated with BC and may be used as predictor and/or prognostic parameters for BC prospection. Our results concerning the prognostic viability of FGFR3 mutations are consistent with those of [24] who indicated that FGFR3 mutations, were associated with a favorable prognosis in patients with urothelial carcinoma compared with wild type, and also are in parallel to those of [8] as they concerned the prognostic utility of FGFR3 mutations and reported that FGFR3 mutations are identified in low-grade tumors and can potentially be used as prognostic biomarker in BC patients. However, our results are completely

different from those of [25], in which they indicated that the FGFR3 mutation is comparatively less common in patients with muscle-invasive BC at the time of diagnosis and has not been established as a prognostic biomarker in advanced BC.

Our findings confirm that FGFR3mutations are associated with tumors of low grade and stage and that there is a significant decrease in the prevalence of mutations as grade and depth of invasion increase. Highest prevalence was found in GII for exon 7 and 10 as the frequency was 72 (73.5%) and 56 (68.3%), respectively, while the opposite was found in exon 15 as the highest prevalence was found in GIII 10 (71.4%). The previous results suggest a potential prognostic value for mutations of exons 7 and 10 and they may help in the early detection of BC.

Regarding the stage of the tumor, highest prevalence was found in T2 for both exons 7 and 10 with frequency of 65 (66.3%) and 53 (64.6%), respectively, while for exon 15, the frequency was 7 (50.0%) in T1 and the same in T2. Our results concur [26]'s results as they reported that analysis of the occurrence of FGFR3 mutations with respect to tumor grade revealed the presence of mutation in (62% of the cases) low-grade tumors and in only (26% of the cases) high-grade tumors. Meanwhile, there is a contradiction between results regarding tumor stage as they reported a negative association in the distribution of FGFR3 mutations according to tumor stage. Our findings are in agreement with those of [8] who indicated that FGFR3 mutations were more frequently identified in low-grade tumors and the occurrence of FGFR3 mutations with respect to tumor stage revealed the presence of FGFR3 mutation in low-stage tumors than high-stage tumors, while [27] published data showing no significant correlation between the presence of FGFR3 mutations and tumor grade or stage in their study.

Notably, the prevalence of mutations in this study has been evaluated as a marker for recurrence and survival in BC and it was significantly associated with recurrence and survival time of patients for all exons and as a consequence for the previous results, FGFR3 mutations could be used as a prognostic marker for BC recurrence using urine samples. Our results provide novel evidence that identification of molecular marker capable of predicting the risk of recurrence will certainly help in better clinical management of BC patients.

Furthermore, wild patients have a lower risk of recurrence than those harboring a mutation. Our results match those of [28] which revealed a significantly higher rate of recurrence in patients harboring FGFR3 mutation compared with FGFR3 wild-type. Conversely, a study by [29] reported that BC recurrence was more common in wil-dtype patients rather than those harboring FGFR3mutation.

Interestingly, Chi-squared test identified CIS as significant predictors for BC especially for mutants in exon 7 and 10 and this was in agreement with Van Rhijn *et al*. [30] as they reported that FGFR3 mutation status and CIS were significant for predicting progression on univariate and multivariate analysis.

We have no idea about when NMIBC would develop into MIBC, so an effective marker was urgent to further predict the progression of BC. Accordingly, the present study clarified a highly significant association between wild BC patients and both of tumor progression and tumor invasiveness with protection from BC of 0.1(0.04–0.4) and p = 0.001. Accordingly, mutations did not predict neither tumor invasiveness nor progression. A study by Van Rhijn *et al*. [30] who examined the distribution and clinical outcome of FGFR3 alterations in 132 patients with primary pT1 BC, they published results contrary to our results, they reported that FGFR3 mutation status was a significant prognostic factor for progression. While [31] demonstrated a different result, in which FGFR3 mutation status did not have prognostic significance in terms of tumor recurrence or progression.

Student t-test identified significant association between mutation prevalence and survival time of mutant patient in exon 7, 10, and 15 with mean survival time of 31.4 ± 8.5, 32.4 ± 8.6, and 38.5 ± 1.6, respectively. These findings revealed that the prevalence of mutation may extend the life time of the mutant patient than the wild one and these mutations may act as significant predictors of survival time of BC patients. Our results are along with those of [32], in which they strongly supported the notion that FGFR3 mutations were associated with good prognosis and better overall survival (OS) in muscle invasive BC.

Kaplan–Meier analysis revealed a significant association between mutant patients in exon 10 and the mean time to survive which was 32.375 months with 95%C.I. of (28.938–35.812) and p = 0.031, while no significant association was observed regarding exon 7 and 15. These results revealed that patients with FGFR3mutations demonstrated better free survival compared with wild patients. The results of the present study were supported by [33] who reported that patients with FGFR3 mutations in the overall cohort had improved survival time compared with patients without FGFR3 mutations and are consistent with those of [34] who found that FGFR3 mutations were significantly associated with a favorable prognosis, with improved longer disease specific survival compared with wild type, in patients with urothelial carcinoma, while [28] reported that mutations failed to independently predict survival either in the whole group of BC patients or in the analyzed tumor subgroups.

ANOVA test was used to analyze the association between the mutant patients and tumor grade and stage, the results revealed the presence of significant association between mutants in exon 7, 10, and GI patients. Regarding tumor stage, an inverse association was detected between the tumor stage T1 of mutant patients in exon 7 and the survival time. From

these associations, we can confirm that survival time decreases in mutant patients as tumor grade gets higher and in advanced stage. Furthermore, [35] published similar results which revealed that mutant patients with the early stage pTa and pT1 and low-grade G1 and G2 bladder tumors had a significantly better OSwhen compared to mutant patients with late stage pT2 and pT3 and high grade G3 of the tumor.

Conclusion

Our results showed that the mutation of exons 7, 10, and 15 of FGFR3 gene may be used as predictor and/or prognostic parameters for BC prospection. These mutations are independent prognostic factors for tumor recurrence and the genetic alternation of FGFR3 could be used for prediction of survival time of BC patients.

Ethics Approval and Consent to Participate

This research work was approved by the ethical committee of Theodor Bilharz Research Institute, Cairo, Egypt according to the regulations adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964.

Availability of Data and Materials

All data and source of used materials are available up-on request.

Authors' Contributions

SM: Suggests the idea of the current work and was responsible for the major part of molecular biology techniques and writing the manuscript. TA: Was responsible for the histopathological and immunohistochemical study of the research. OH: Was responsible for the FISH study of the research. FK: Share in doing the molecular biology technique. GS: Collection of data, tabulation of results. KE: Was responsible for clinical diagnosis of patients, doing the cystoscopic examination, and collecting the biopsy and urine samples of the study.

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