ORIGINAL ARTICLE

DIFFERENTIAL EXPRESSION OF FGFRs SIGNALING PATHWAY COMPONENTS IN BLADDER CANCER: A STEP TOWARD PERSONALIZED MEDICINE

Ousati Ashtiani Z[1,2], Tavakkoly-Bazzaz J[2,*], Salami SA[3], Pourmand MR[4], Mansouri F[5,6], Mashahdi R[1], Pourmand G[1,*]

*Corresponding Author: Professor Gholamreza Pourmand, Urology Research Center, Sina Hospital, Tehran University Medical Sciences, Hasan Abad Square, Tehran, 113746911, Iran. Tel: +98-216-634-8560. Fax: +98-216-634-8561. Email: pourmand@tums.ac.ir and/or Associate Professor Javad Tavakkoly-Bazzaz, Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Poursina Street, Tehran, 1417613151, Iran. Tel: +98-218-895-3005. Fax: +98-218-895-3005. Email: tavakkolybazzazj@tums.ac.ir

ABSTRACT

Variations Improper activation and inappropriate expression of fibroblast growth factor receptors (*FGFRs*) in cancer suggests that they can act as therapeutic targets. Fibroblast growth factor receptor inhibitors are currently employed in clinical trials of different cancers. Regarding the essence and the importance of the personalized medicine, mainly mirrored by remarkable inter-individual variations in different populations, we aimed to perform a pilot study to address *FGFR1* and *FGFR3* expression levels and their correlation with the clinicopathological features in Iranian patients with bladder cancer (BC). Paired tumor and adjacent non tumor tissue samples along with their clinico-pathological parameters were obtained from 50 cases diagnosed with BC in different stages and grades. The mRNA expressions of *FGFR1* and *FGFR3* in tissue samples were determined by real-time polymerase chain reaction (real-time PCR). The expression levels of *FGFR3* were significantly higher in tumor tissues when compared to adjacent normal tissues ($p = 0.007$), regardless of the stages and grades of the tumor. Over expression was associated with cigarette smoking ($p = 0.037$) and family history for cancer ($p = 0.004$). Decreased expression of *FGFR1* was observed, remarkably evident in high-grade tumors ($p = 0.047$), while over expression was detected in low-grade samples. This pilot study clearly suggests that in Iranian BC patients *FGFR1* and *FGFR3* expression patterns are different, and also highly distinctive with regard to the tumor's stage and grade. Such particular expression patterns may indicate their special values to be employed for interventional studies aiming targeted therapy. Further studies with a larger sample size are needed to validate our results.

Keywords: Bladder cancer (BC); FGFR signaling; *FGFR1*; *FGFR3*; Targeted therapy.

INTRODUCTION

Bladder cancer (BC) is one of the most common types of urinary system cancers in men, and with less frequency, in women worldwide [1]. It is a complex disease resulting from both genetic and environmental factors. Aberrations in different genes' structure and function, molecular derangements, and several environmental factors have been found to play a crucial role in the development of BC. Age, increased body mass index (BMI), occupation, unhealthy diet, and some drugs can increase the risk of BC development [2]. The epigenetic mechanism also involves in BC tumorigenesis [3]. Blood cancers are generally classified as non muscle invasive with low-grade (pTa/T1) and muscle invasive (pT2-4) that are frequently high-grade tumors [4].

Regulation of different processes during cell cycles, including cell growth, differentiation, cell movement, and apoptosis are orchestrated by diverse signaling pathways. When the above mentioned processes are dysregulated secondary to changes in a key genetic element of cellular homeostasis, tumorigenesis can be the outcome. The fi-

[1] Urology Research Center, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

[3] Department of Biotechnology, University of Tehran, Tehran, Iran

[4] Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

[5] Department of Medical Immunology and Genetics, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran

[6] Cellular and Molecular Research Center, Urmia University of Medical Sciences, Urmia, Iran

broblast growth factor receptor (FGFR) signaling pathway has been receiving growing attention as one of the major contributors in cell cycle regulation that in turn, introduces this specific molecule as a potential drug target for cancer therapy [5-7].

The mammalian fibroblast growth factors (FGFs) are a family of growth factors, consisting of 18-22 members that play an essential role in multiple physiological events such as angiogenesis, wound healing, embryonic development, and various endocrine signaling pathways both in health and disease. Fibroblast growth factors signaling dysregulation is evidently present in a considerable number of BC cases [8]. A subfamily of receptor tyrosine kinases (RTKs) named fibroblast growth factor receptor family comprises of four members (FGFR1-4). They are activated by binding to their ligand FGFs, which results in kinase activation. Different changes exemplified by *FGFRs* mutations and translocations, as well as alterations in mRNA splicing and gene amplification of FGF/FGFR pathway and protein expressions levels have been documented in different cancers [9-14]. Aberrations of the FGFR signaling pathway can activate downstream pathways, PI3K/AKT, MAPK signaling cascade, those which contribute to tumor progression. The *FGFR1* and *FGFR3* mutations and over expression have been reported in BC [15-18], while *FGFR3* alterations were significantly involved in the pathogenesis of urothelial carcinoma (UC) as a whole. However, its clinicopathological implications and significance have not so far been well addressed, especially in the case of muscle-invasive BCs [19]. In contrast to the non muscle invasive UC, where the *FGFR3* is frequently mutated or overexpressed, in muscle invasive forms the incidence of *FGFR3* mutation and mRNA/protein expression changes remain unknown [20]. The *FGFR1* gene expression alteration is also related to certain cancers [8,9, 14]. More notably, a recent study using next generation sequencing in advanced BC has demonstrated a gene fusion of *FGFR1* and *NTM* (*FGFR1-NTM*) [21].

Molecular genetic studies on *FGFR1* and *FGFR3* have revealed the role of these gene changes in different cancers and their value in molecule-targeted therapy. The present study was conducted because of a significant heterogeneity in response of the BC cells to FGFR inhibitors that highlights the importance of the personalized medicine, and also with regard to the remarkable inter-individual variations between different populations. For the first time, this study designed to evaluate *FGFR1* and *FGFR3* expressions at the mRNA level, and their associations with grade, stage and other clinicopathological features in Iranian subjects with BCs.

MATERIALS AND METHODS

Patients and Tissue Samples. Paired samples, both bladder tumor and adjacent normal tissue were obtained from 50 Iranian individuals who underwent transurethral bladder tumor resection or radical cystectomy at two university teaching hospitals (Sina and Imam Khomeini Hospitals) in Tehran, Iran. Bladder tumor and non tumor samples from a standard distance were rapidly frozen in liquid nitrogen following collection and stored at –80 °C until subsequent RNA extraction.

Of the 50 patients, 43 were males and seven were females. The median age was 66 years, ranging from 33 to 84 years. None of the patients received any treatments, such as Bacillus Calmette-Guerin (BCG) therapy, chemotherapy, which might alter the situation of the FGFR signaling pathway in terms of its status and activity. Clinicopathological information including grade, stage, lymph node metastasis, age, gender, smoking, alcohol use, family history of cancer, was provided for all subjects. In this research, written informed consent was signed by all participants, after being informed about the goals of the study. This study was approved by the Research Review Board and also the Ethics Committee of Tehran University of Medical Sciences (TUMS), Tehran, Iran.

Total RNA from both tumor and adjacent non tumor tissues were isolated using TriPure Isolation Reagent (Roche Life Science, Mannheim, Germany) according to the manufacturer's protocol. The quality and quantity of extracted RNAs were measured by the absorbance ratio at 280/260 nm using NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). In order to remove possible DNA contamination from RNA, DNase I treatment was performed. The cDNA was synthesized from 1 μg RNA by oligo dT, Random 6-mer and reverse transcription Enzyme using PrimeScript™ RT reagent kit (Takara, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. It was designed to perform optimized reverse transcription-polymerase chain reaction (RT-PCR). Thermal Cycler (Senso Quest GmbH, Göttingen, Germany) was used for the incubation reaction mixture at 37 °C for 15 min. and 85 °C for 5 seconds. The cDNAs were stored at –20 °C until further use.

For real-time PCR, specific sets of primers were designed for *FGFR1*, *FGFR3* and *GAPDH* as housekeeping genes. All amplicon lengths for real-time PCR were less than 200 bp long. Primer sets were checked by primer-BLAST and Oligoanalyzer software (https://eu.idtdna.com/ calc/analyzer). Table 1 shows the 5'>3' sequence of the primers and amplicon lengths.

Table 1. List of primer sets for real-time polymerase chain reaction.

Primers	Sequences (5'>3')	Amplicon Size
FGFR1-F FGFR1-R	GAA GAC TGC TGG AGT TAA TAC C TCT TCC AGG GCT TCC AGA AC	159
FGFR3-F FGFR3-R	CCA CTG TCT GGG TCA AGG AT AGG ATG GAG CGT CTG TCA C	180
GAPDH-F GAPDH-R	ATC CTG GGC TAC ACT GAG C CAC CAC CCT GTT GCT GTA G	159

F: forward; R: reverse.

Table 2. Clinicopathological characteristics of the study subjects.

Clinicopathological Parameters	n	%
Age (years): ≤66 >66	 26 24	 52.0 48.0
Sex: males females	 43 7	 86.0 14.0
Stage: I II III IV	 16 20 10 4	 32.0 40.0 20.0 8.0
Grade: low high	 14 36	 28.0 72.0
Tumor type: non invasive muscle muscle invasive	 15 35	 30.0 70.0
Family history: bladder cancer other cancer no cancer	 8 6 36	 16.0 12.0 72.0
Smoker: ≤10 >10 non smoker	 2 32 16	 4.0 64.0 32.0
Occupational exposure	27	54.0

Real-time PCR was performed using SYBR Premix EX Taq™II (Takara). The reaction mixture was prepared according to the manufacturer's protocol. The cycling conditions were: 10 seconds at 95 °C (Takara Master does not need longer hold), followed by 40 cycles at 95 °C for 10 seconds and 60 °C for the 20 seconds. Amplification reactions were performed in triplicate for each sample. A melting curve was obtained following amplification. No template control (NTC; nuclease-free water) was included in each run. The quantitative PCR (qPCR) analysis was completed using Rotor-Gene Q (Brisbane, Queensland, Australia). Cycle threshold (ct) values were collected for the genes of interest and glyceraldehyde 3-phosphate dehy-

drogenase as housekeeping gene during the log phase of the cycle. Results were normalized to the GAPDH as a reference gene. Agarose gel electrophoresis was used to determine the specificity of the RT-PCR reaction products. Gene expression data analysis was carried out using the $2^{-\Delta\Delta CT}$ method according to the following formula: $\Delta ct_1 = ct_{target} - ct_{housekeeping}$, $\Delta ct_2 = ct_{normal} - ct_{housekeeping}$, $\Delta\Delta ct = \Delta ct_1 - \Delta ct_2$.

Statistical Analysis. Statistical analysis was performed by the the Statistical Package for Social Sciences (SPSS) version 21 (IBM SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to assess the normality of quantitative data. Comparison of normalized expression between tumor and non tumor tissues was done using the parametric *t*-test for FGFR3 and nonparametric Mann-Whitney test for FGFR1. A multivariate linear regression analysis was performed to find the relationship between expression and clinicopathological parameters as independent variables by a stepwise method in the model. In these tests, a *p* value of ≤0.05 was considered to indicate a significant difference.

RESULTS

Patient Characteristics. This study comprised 50 BC patients including 43 (86.0%) men and seven (14.0%) women. Analyses of the subjects showed that 36 (72.0%) cases had high-grade and 14 (28.0%) had low-grade tumors. Their median age was 66 years (range 33-84 years). There were 34 patients (68.0%) with a smoking habit, of which 94.0% (32/34) had been cigarette smokers for ≥10 years and 20.0% (10/50) were opium addicts. Of these subjects, 16.0% (8/50) had diabetes and 46.0% (23/50) showed cardiovascular and/or respiratory diseases. The rate of occupational exposure was about 54.0% (27/50). Table 2 shows the demographic and clinicopathological characteristics of all subjects.

Increased mRNA expression of F*GFR3* was observed in 92.0% (46/50) of tumors. Analysis by a multi variable regression method with a stepwise selection variable demonstrated that there was significant association between

increased expression of *FGFR3* and cigarette smoking (*p* = 0.037) and family history of cancer (*p* = 0.004) (Table 3). We could not find a significant relationship between FGFR3 mRNA over expression and age (*p* = 0.094) and other relevant clinicopathological parameters including stage, grade and types of the tumor. The comparison of normalized expression in tumor and non tumor tissues showed significant difference (fold change 5.7; *p* = 0.01) (Figure 1).

Unexpectedly, FGFR1 mRNA expression in our BC cases was decreased in 60.0% (30/50) of samples. The majority of high grade tumor (75.0%) (27/36) reflected a significant association with decreased level of FGFR1 mRNA expression (*p* = 0.047). About 64.2% (9/14) of low-grade tumors showed increased expression of *FGFR1*. There was also a significant association between normalized expression in tumor *vs.* non tumor tissue (fold change –4.5; *p* = 0.01) (Figure 2). Other clinicopathological parameters did not represent any significant difference with *FGFR1* expression.

DISCUSSION

This is the first study that demonstrates FGFR1 and FGFR3 mRNA expression levels in Iranian patients with BCs. The FGF/FGFR pathway is one of the central mecha-nisms that govern differentiation, proliferation, survival and many other issues of cellular characteristics. Large number of evidence supports the appropriate functionality of FGFs-FGFRs systems is dysregulated at any point within their signaling cascade that could frequently lead to cancer development *via* activation of the downstream pathway [22].

The *FGFs* and *FGFRs* changes at different levels featured by gene mutations, gene fusions and aberrant expressions or amplification have been identified in the progression of UCs [8,21]. Such aberrations in *FGFR3* are one of the most common molecular events in UCs, but some of them such as gene amplification are relatively rare [23,24]. Preclinical studies weighed the applicability of *FGFR3* for targeted therapy [25]. In this pilot study, FGFR3 mRNA over expression was observed in the major-ity of all subjects (shaped mainly by high grade or muscle invasive tumors) (92.0%), while most of the previous stud-ies were conducted in cases with non muscle invasive tumors [20, 26]. The frequency of FGFR3 mRNA over expression between the subjects of the present study was clearly higher than that of previous reports in BC [20]. Analyses of our data showed that FGFR3 mRNA over expression was associated with smoking and family history (*p* = 0.037, *p* = 0.004, respectively). This FGFR3 tumoral over expression was not related to the other clinicopatho-

Table 3. Multivariate linear regression analysis of FGFR1 and FGFR3 expressions with clinicopathological variables.

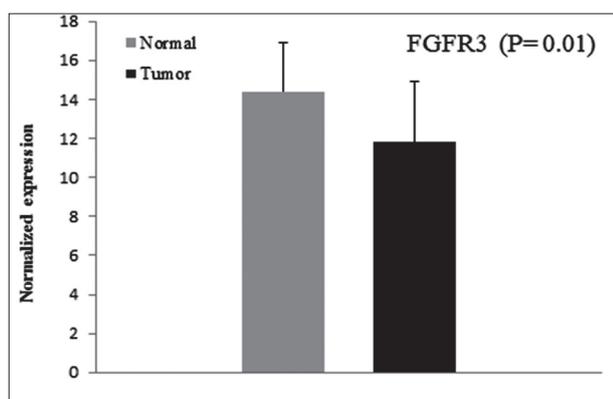| Relative Expression	Variable(s)	Undersized Coefficients B	Standard Error B	*t*-Test	*p* Value
FGFR1	no significant variable				
FGFR3	(constant) family history of BC cigarette smoking	–28.908 14.266 11.568	11.031 4.125 4.968	–2.621 3.458 2.329	0.021 0.004 0.037

BC: bladder cancer.

Figure 1. Normalized expression of FGFR3 in cancerous and normal tissues.
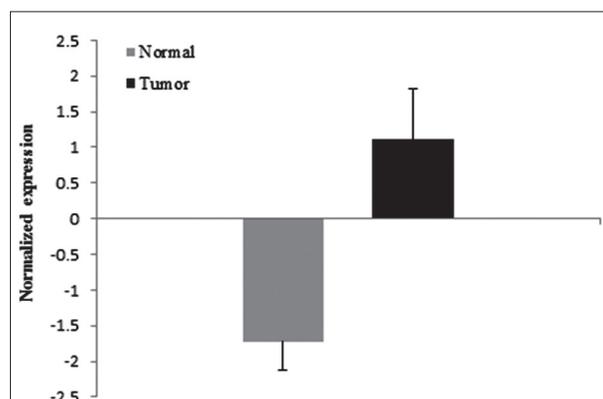

Figure 2. Normalized expression of FGFR1 in cancerous and normal tissues.

logical parameters including tumor grade or stage. Since up- and down-regulation of *FGFRs* have been found in the context of different cancers [10,19,28,29], it is suggested that the potential oncogenic and tumor suppressive influencing *FGFRs* can be implemented by either its up- or down-regulation according to the nature and stages/grades of the tumors. Mainly mediated by *FGFR3* gene mutations, over expression of FGFR3 mRNA and protein levels have been evident frequently in different cancers, which may suggest more clearly its oncogenic properties [10,22,27]. In the current study, increased expression of *FGFR3* in most of the tumor tissues, regardless of their grade, stage and type, suggest the importance of the *FGFR3* role as a driving member for tumor growth and progression in Iranian BC subjects.

In terms of *FGFR1* expression patterns, to some extent, our results are not consistent with previous reports. We observed decreased mRNA expression of *FGFR1* in 60.0% of cases, of which the majority of them had high-grade BC ($p = 0.01$), while previous studies found an increased expression of both mRNA and protein in mostly non invasive BC [9,20,24,29]. However, in another study that was conducted on an invasive form of BC, the level of FGFR1 was significantly up-regulated [18]. This discrepancy may be partly attributed to geographical and methodological parameters including technical issues as well as histopathological heterogeneity within individual tumor type and also proportion of case within various grade and stage classes.

While the pivotal role of the *FGFR1* gene in the development of different types of cancer is suggested by several studies, it seems the underlying mechanism for *FGFR1* gene involvement or the pattern of its dysregulated expression is not fixed. For example, it is reported that the main reason for *FGFR1* up-regulation in breast cancer is the amplification of the *FGFR1* gene at the genome level that could be evidenced by high *FGFR1* expression level in breast tumors [30], whereas we mentioned before, it seems that the amplification of *FGFRs* genes is not the case in BC. In addition to BC, the reduced expression of *FGFR1* is also evident in some other types of cancers, such as parathyroid cancer [14]. Such decreased expression patterns, which may suggest a potential tumor suppressive role for *FGFRs*, was also reported for *FGFR2* in several cancers including bladder, liver, salivary gland and prostate; but the exact mechanism remains unknown [22].

However, it is so complicated to find out which member of the FGFRs system is playing the central role in cancer. Cheng *et al.* [29] stated that *FGFR3* plays a more important role than *FGFR1* in stimulating BC tumor cell proliferation. Our data indicates that reduced mRNA expression of *FGFR1* is associated with high-grade tumors, contrasted by its increased expression in low-grade tumors. This would suggest that although *FGFR1* is less important than *FGFR3* in BC cell proliferation, up-regulation of *FGFR1* expression can be considered as an early event, the same as *FGFR3*, in the initiation and development of BC that was also raised by some previous reports [18,31]. In this regard, increased expression of *FGFR1* and *FGFR3* proteins are reported in the early stages of non small cell lung cancer [13].

In addition, it seems that *FGFRs* act differentially depending on the tumor's type that could explain that contradictory results may be partly due to the cell type specificity. For instance, in cervical cancer, expression of *FGFR1*, *FGFR2* and *FGFR4* were higher in cancerous tissues, whereas *FGFR3* was higher in non cancerous tissues [27]. In addition to cell specificity, alternative splicing of the extracellular fragment of *FGFR1-3* may be partly accountable for this discrepancy. Alternative splicing can result in the formation of isoforms, which in turn, change ligand-binding specificity and the switching of the cross-talk between isoforms. This, in turn, causes alterations in FGFR signaling leading to influences on downstream signaling cascades. It has been reported that two splice variants of *FGFR1* gene called FGFR1α and FGFR1β were expressed at similar levels in normal urothelial cells, but expression level of FGFR1β in tumor cells were higher than in controls. They also presented that FGFR1β: FGFR1α ratio was significantly increased in relation to tumor stage and grade (31). On the other hand, the epigenetic process alterations are thought to influence gene expression largely at the transcription level [32], it may be considered as a reason for this inconsistency in different studies.

To the best of our knowledge, this pilot study is the first report examining the expression of two components of FGFR signaling pathway (*FGFR1*, *FGFR3*) in Iranian BC subjects in order to find out whether they can be used as a molecule for targeted interventions. The present study showed that lower mRNA expression of *FGFR1* was significantly associated with high-grade tumors and in low-grade tumors increased expression was observed. Our results suggest that the expression mode of *FGFR1* is somehow related to the cancer grade. With regard to *FGFR1* over expression in low-grade tumors and *FGFR3* over expression in both high- and low-grade tumors, it is probable that these components of FGFR signaling cascade may be considered distinctly as potential candidates for targeted therapy.

However, under individualized medicine strategy for BC in the future, it seems that FGFRs could provide attractive targets for therapeutic interventions. Because of the heterogeneity between different populations, such objective requires a more comprehensive understanding on the role of FGFRs in BC, coupling with the data on larger sample sizes of the cases and controls. Finally, it is very crucial to remember that the inter-individual phenotypic variation at the cellular and molecular level is not solely dictated by genetic impressions. Accordingly, epigenetic issues, which themselves are influenced by environmental factors, should be taken into account to unravel the reality behind these observations.

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