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T375c Mutation of FGFR3 Gene in Bladder Epithelial Cells of Patients with Bladder Cancer

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ABSTRACT

BACKGROUND AND AIM: The FGFR3 gene plays an important role in regulating growth, differentiation and angiogenesis. The genetic changes of the FGFR3 gene are one of the most important factors in bladder cancer. Active mutations of FGFR3 have been observed in about 70% of non-muscle-invasive bladder cancers. The present study was conducted to evaluate T375C mutation of FGFR3 gene in genomic DNA extracted from bladder epithelial cells and the risk of bladder cancer.

METHODS: In this case-control study, 100 healthy individuals and 100 patients with bladder cancer who referred to health centers in northern Iran were selected using convenience sampling after confirmation of the diagnosis of this disease after pathological examinations of the biopsy. Differences in the frequency of allele and genotype were evaluated. After urine centrifugation, genomic DNA was extracted from cell precipitate using commercial kit. The status of T375C mutation of FGFR3 gene was studied using specific primers and tetra-ARMS-PCR technique.

FINDINGS: The frequency of genotypes was 94% (TT), 4% (TC) and 2% (CC) in the case group versus the absence of the TC and CC genotypes in the control group. There was a significant relationship between allelic and genotypic distribution ($p = 0.0124$ and $p = 0.0192$, respectively).

CONCLUSION: The results indicate that the T375C mutation of the FGFR3 gene may be a genetic susceptibility factor to bladder cancer.

KEY WORDS: *FGFR3 protein, genetic changes, bladder cancer.*

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Introduction

Bladder cancer is the fourth most common cancer in men and the ninth most common cancer in women (1). It is also the second most common cancer of the genitourinary system (2). About 70% of bladder cancer cases are non – muscle – invasive, and 30% remaining are muscle – invasive tumors (3). The most important risk factors known to date for bladder cancer include cigarettes and occupational exposure to certain chemicals, such as aromatic amines (4). Bladder cancer patients are diagnosed through urinary cytology, kidney ultrasound, and cystoscopy (5). High recurrence rates in patients with non – muscle – invasive bladder cancer (NMIBC) and the risk of progression to invasive – muscle bladder cancer (MIBC) require frequent monitoring (6). The need for hospital visits and follow-up visits, high costs, and the use of aggressive methods make it the most costly cancer therapeutically (7). To improve this condition during the so-called liquid biopsy (8), research is focused on the development of tests that are specifically used to detect molecular changes and are very common in these patients (9). Gene-based urine biomarkers have a higher sensitivity to urine-based diagnosis. That's because they detect cancer-related changes that are less likely to be affected by inflammatory conditions and other benign diseases. In addition, they are easily accessible and not invasive (3). Therefore, non-invasive tests for bladder cancer and other cancers will improve the management of the disease by reducing the incidence of aggressive methods and early identification of patients. Bladder cancer is not usually hereditary but due to accumulation of somatic mutations in the bladder cells over time (10), identifying genetic changes in the bladder carcinoma is important in our understanding of the pathogenesis of this disease and will be helpful in designing improved strategies for prevention, diagnosis, prognosis and treatment. FGFR3, HRAS, and TERT are among the genes involved in bladder cancer tumorigenesis. Among these, FGFR3 is one of the most frequently mutated genes in bladder cancer (11). FGFR3 oncogene is a receptor tyrosine kinase growth factor with recurrent mutations (60 – 70%) in non – muscle – invasive bladder cancer and mutations with less frequency (0.2 – 20%) in muscle – invasive bladder cancer (12). The mRNA binding mechanisms also produce different receptor isoforms, including FGFR3b and FGFR3c (1). Active mutations of FGFR3 have been observed in about 75% of bladder papillary tumors (13). Recent studies have shown the association between FGFR3 mutations in low – grade non – muscle – invasive bladder cancer (LG – NMIBC) with a high risk of recurrence (14). Obviously, FGFR3 plays an important role in the development of low – grade non – muscle – invasive bladder cancer (LG – NMIBC). Although the phenotypic outcomes of the FGFR3 mutation and the details of the implications of the active receptor signaling in the urothelium are unknown at present, it is anticipated that this receptor play an important role as a biomarker and therapeutic goal (7). FGFR3 is related to growth regulation, differentiation, migration, wound healing and angiogenesis (15).

This gene is located on the chromosome 4p16 and has 19 exons and 18 introns (16). This protein participates in a variety of diseases, such as achondroplasia, skin diseases, bladder cancer, cervical cancer, myeloma, and cartilage growth abnormalities (17). Genetic somatic mutations are one of the most important factors in tumor progression and progression of bladder cancer (18). The active mutations of FGFR3 and excessive expression of the wild-type receptor are two mechanisms associated with tumorigenicity (19). FGFR3 mutations activate the Ras-MAP kinase signaling pathway and phospholipase CY (PLCY), resulting in uncontrolled cell proliferation (20). 97% of the active mutations in FGFR3 in bladder cancer occur in exons 7, 10, and 15. Mutations of exons 7 or 10 cause dimerization and independent activation of the ligand (15).

Common mutations in exons 7 and 10 include S249C (61%), T375C (19%), R248C (8%) and G370C (6%) (21). The mutations in exons 7 and 10 lead to changes in the extracellular region and the transmembrane region, respectively (22). A study in Denmark showed that the increase in mutated FGFR3 in DNA of urine and plasma showed progression and metastasis in bladder cancer. Of 65 tumor samples studied by them, 6 samples had T375C mutations (23).

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In a study by a research team in the United States, mutations in the FGFR3 gene were observed in 64% of primary bladder tumor samples, and the T375C mutation occurred in 27 samples out of 257 bladder cancer samples. Their results showed that the FGFR3 gene could be used as a biomarker and a therapeutic goal (24). Considering the role of FGFR3 in tumor development and the importance of using noninvasive methods, this study was conducted to investigate T375C mutation of FGFR3 gene in urinary epithelial cells in patients with bladder cancer.

Methods

After being approved by the Ethics Committee of the Islamic Azad University of Rasht (the code of ethics IR.IAU.RASHT.REC.1396.130), this case-control study was conducted among 100 healthy subjects (as controls) and 100 patients with bladder cancer who were referred to Shahid Beheshti Hospital in Babol, Razi Hospital in Rasht and Imam Hospital in Sari using convenience sampling method. The inclusion criteria were urine cytology, mass diagnosis in the bladder by ultrasound in patients, lack of performing invasive procedures such as biopsy or surgery within one month before sampling, lack of another chronic or malignant disease, and not receiving treatments such as chemotherapy or radiotherapy.

Urine samples were collected from patients during a six-month period from February 2017 to July 2017 after obtaining informed written consent. Urine samples were collected before surgery. At the same time, data collection form was also provided to the subjected and they completed the form. In this form, the information such as the symptoms of the disease, and history of previous diseases were introduced. Among the collected samples, only the samples whose bladder cancer was confirmed in pathological examinations following tissue sampling (biopsy) were examined. Samples were classified according to the pathology results from pathology centers based on the depth of invasion, degree of invasion, age and gender. After collecting urine samples (20 – 50 ml), they were centrifuged at 5000 rpm for 10 minutes, then the supernatant was discarded and 500 µl of ethanol 70% was added to the sediment, and were transferred to -20 °C freezer for further experiments and genetic studies. DNA extraction from urine cell sediment was performed by ZPZ Company Kit in accordance with the protocol of the company. In order to assess the extracted DNA quantitatively, a biophotometer was used. For qualitative assessment, the extracted DNA was electrophoresed on 1% agarose gel.

The tetra primer PCR method was used to determine the T375C mutation of FGFR3 gene. In this method, four primers are used. The designing of the primers used in this study was done by Oligo Analyzer tool and the primers were synthesized by the Copenhagen Company. The properties of primers and their melting temperature were also evaluated by the BLAST program. The sequence of primers designed to determine the genotype of region rs: 121319485 is presented in Table 1.

Table 1. Primer sequence used to amplify codon 375 exon 10 of the FGFR3 gene

Product length	Primer Sequence (5' to 3')		Codon
240 bp	F: TCTGGCCCTCTAGACTCAC	Interior	375
	R: CGTAGCTGAGGATGCCTGAAC	Primer	rs: 121913485
664 bp	F: TTCTCTCCTTGCACAACGTCA	Exterior	
	R: CGTAGCTGAGGATGCCTGAAT	Primer	

Each vial was prepared from a PCR reaction in 25 µl final volume containing 5 µl genomic DNA, 12.5 µl Master Mix (AMPLICON Co.), 6.5 µl sterile distilled water and 1 µl of a mixture of forward and reverse primers. The polymerase chain reaction was then performed using BioRAD. The optimal PCR conditions for the T375C mutation included an initial denaturation of two strands at 95 °C for 4 minutes, with 30 cycles,

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each including 45 seconds denaturation at 95 °C, 35 seconds primer binding at 60°C, 1 minute complementary strand extension followed by primers at 72 °C, and then at the end of the reaction, 5 minutes complementary strand extension at 72°C. The PCR products were stored in a -20°C freezer for further analysis. In order to determine the allelic and genotypic distribution, and the relationship between genotypic association and hereditary models, MedCalc program version 15.8 and Chi-square test were used. In addition, $p<0.05$ was considered significant.

Results

In this study, of 100 patients with bladder cancer, 89 patients (89%) were male and 11 patients (11%) were female with a mean age of 61.5±21.5 years. Of 100 healthy people, 65 people (65%) were male and 35 people (35%) were female with a mean age of 53±15.5 years. In the case group, 81 patients (81%) had grade 1 tumors, 10 patients (10%) had grade 2 and 9 patients (9%) had grade 3 tumors. In addition, 96 patients (96%) had non – muscle – invasive tumors and 4 patients (4%) had muscle – invasive tumors (Table 2). Identification of T allele with fragment length of 664 bp and C allele with fragment length of 240 bp was done on 1.5% agarose gel (Fig 1).

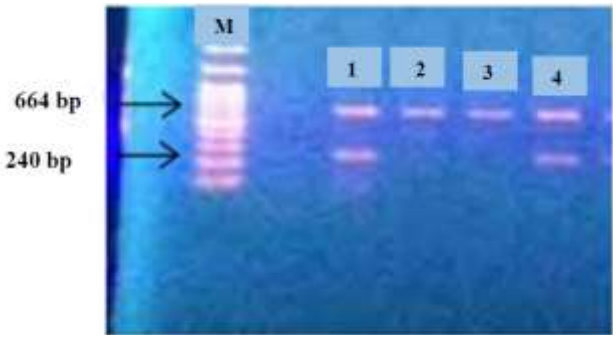

Figure 1. Results of PCR of T375C mutation in samples 1, 2, 3, 4 on 1.5% agarose gel. M is DNA marker (100 pb). The arrow shows the location of the fragment.

After analyzing the data from the PCR reaction, it was found that 6 (6%) of the samples had T375C mutations, which were only observed in men. These mutations were also present in non – muscle – invasive samples. The mutation was not observed in the healthy group (Table 2). Of 100 patients with bladder cancer in this study, 94 patients (94%) were heterozygous genotypes TT, 4 patients (4%) were heterozygous genotypes TC and 2 patients (2%) heterozygous genotypes CC. In control group, all 100 patients (100%) were heterozygous genotypes TT. The results of MedCalc program showed a statistically significant relationship between case and control group ($p=0.0192$) in terms of genotype. The frequency of T allele in control and case groups were 100% and 96%, respectively, and C allele was only present in the group of patients with a frequency of 4%. There was a significant relationship between the two groups in terms of distribution of allele ($p=0.0124$) (Table 3).

Table 2. Results from the study population

	N	T375C	Women	Men
Patient	100	6	11	89
Control	100	0	35	65
Non-muscle invasive	96	6	10	86
Muscle invasive	4	-	1	3
Classification				
Grade 1	71	5	10	71
Grade 2	20	1	1	9
Grade 3	9	–	–	9

Table 3. Analysis of genotypic and allelic T375C mutation of FGFR3 gene between healthy group and group of patients with bladder cancer

Genotype	Patients N(%)	Healthy control N(%)	P-value
TT	94(94)	100(100)	0.047
TC	4(4)	0	0.140
CC	2(2)	0	0.213
Allele			
T	192(96)	200(100)	
C	8(4)	0	0.048

Discussion

The results of the present study revealed the presence of 6 mutations in codon 375 exon 10 of the

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FGFR3 gene in the patients group. The 6 observed mutations leads to the conversion of the amino acid tyrosine to glycine. In addition, the results of statistical studies regarding genotypic and allelic frequency at region rs: 121319485 showed a significant relationship between the control and patient groups (p=0.0192, p=0.0124, respectively). Based on the findings of this study, this study was the first to evaluate the T375C mutation of FGFR3 gene in patients with bladder cancer in Iranian population. Dodurga et al. examined the incidence of FGFR3 mutations in exons 7 and 10 and reported that FGFR3 mutation occurred in the T375C codon in 3 out of 56 bladder cancer cases (1). In a study in the United States, Gust et al. also investigated the mutation and expression of FGFR3 in bladder tumor samples using Real Time PCR and Western Blot, and T375C mutation occurred in 7 samples of the total 153 samples (2). In Canada, Van Rhijn et al. examined the frequency of FGFR3 mutations in 132 samples of pT1 primary bladder tumors by SNaPshot method, the most common mutation in their study was S249C and the T375C mutation was observed in 5 samples (25). In Canada, Kompier et al. also investigated FGFR3 mutations in 257 tumor tissue samples of patients with bladder cancer by SNaPshot method and T375C mutation was found in 27 samples (26). In Japan, Miyake et al. examined FGFR3 mutations in 45 samples of the bladder tumor by real time PCR, and FGFR3 mutations in the T375C codon occurred in 9 cases (37.5%) (27). These differences in mutation frequency may be due to DNA extraction from different samoles, differences in population size, different sampling methods, and different analytical techniques. According to investigations by Traczyk-Borszynska et al. in Poland, the most frequent mutations were FGFR3, S249C and subsequently T375C (28).

On the other hand, the most frequent mutation in FGFR3 identified by Guancial et al. in the United States was T375C and then R248C, while other studies reported S249C as the most frequent mutation in FGFR3 with a lower incidence of T375C (29). Changes in tumor biology are likely to be affected by changes in stage or degree, the study of various ethnic and geographical populations, differences in lifestyle, reproductive patterns and dietary habits. The results of this study showed that T375C mutation of FGFR3 gene has a significant relationship with bladder cancer and is probably associated with susceptibility to bladder cancer.

However, achieving a definitive outcome requires studies with larger statistical community and the rest of the population. It is also suggested that the role of this gene in carcinogenesis of the bladder be assessed in order to evaluate other SNGs of the FGFR3 gene effective in bladder cancer and also to investigate the mutations of FGFR3 in bigger populations and different ethnic groups.

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