Detection of Three Novel Mutations in Exon 7 of FGFR3 Gene in Iraqi Patients with Bladder Cancer

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

Key words: Bladder carcinoma, FGFR3, RFLP, g.1315 delC, g.13529 ins A, g.13510 del A

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

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

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

Approximately 80% of transitional cell carcinomas are confined to the epithelium (pTa, Carcinoma in situ) or lamina propria (pT1) at initial diagnosis, but the remaining 20% invade the muscularis propria (pT2, pT3, pT4). pTa lesions (papillary tumors) are the most common form of bladder carcinoma (Billerey et al., 2001).
More than 50% of primary bladder urothelial cell carcinomas, especially in low-grade and low-stage papillary tumors (Hernandez et al., 2005).

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

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

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

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

Polymerase chain reaction (PCR) for exon 7(120bp)
The exon 7 region of FGFR3 was amplified by PCR using the primers, F 5’ CGGCAGTGCCGTTGTTGTTG3 and R 5’ AGACCCGCGTCTGTTG3 and the condition, initial denaturation 5 minutes at 95 °C, followed by 40 cycle each of denaturation 1 minute at 95 °C, annealing 1 minute at 67 °C, extension 1 minute at 72 °C and a final extension step at 72 °C for 10 minute. PCR products (3 µl) were then separated on 3% agarose gel with a ladder (100bp) and visualized.

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

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

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

Table 1: Bladder cancer and healthy profiles.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy control</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age(years)</td>
<td>58</td>
<td>62</td>
</tr>
<tr>
<td>Mean</td>
<td>30-50</td>
<td>30-86</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>Female</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13</td>
<td>39</td>
</tr>
<tr>
<td>no</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Family history</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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

RELP analysis of FGFR3 exon 7

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

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

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

**FGFR3 exon 7 region/ HaeII enzyme site**

13465 CGGCAGTGGCGGTGGTGTTGAGGGAGGGGGTGCCCCTGAGCGTCATCTGCCCCCAACGAGCGCGCCCAACCAGACGGCGGTG CT 13584.

**FGFR3 exon 7 region/ TseI enzyme site**

1365 CGGCAGTGGCGGTGGTGTTGAGGGAGGGGGTGCCCCTGAGCGTCATCTGCCCCCAACGAGCGCGCCCAACCAGACGGCGGTGCT 13584.
These results showed that the PCR amplified regions of the FGFR3 exon 7 has only one restriction site for each enzymes. The REFLP molecular analysis of FGFR3 exon 7 of patient samples for both enzymes revealed one mutation in one patient include FGFR3 Arginine 248 Cysteine mutation.

**Detection of FGFR3 exon 7 mutations by DNA sequencing**

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

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

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. of mutation in 47 patients / %</th>
<th>Change code</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>g.13515 del C</td>
<td>34 / 72 %</td>
<td>Cys/Cys</td>
<td>Framshift</td>
</tr>
<tr>
<td>GCC&gt;G-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.13510 del A</td>
<td>12 / 30%</td>
<td>Ser/Ser</td>
<td>Framshift</td>
</tr>
<tr>
<td>TCA&gt;TC-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.13529 Ins A</td>
<td>1 / 2%</td>
<td>Ser/Ile</td>
<td>Framshift</td>
</tr>
<tr>
<td>TCC&gt;ATC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Figure (5): Site 13510 del A, nucleotide sequence (forward) in exon 7.
In this study, the REFLP molecular analysis of FGFR3 exon 7 of patient samples for both enzymes revealed one mutation in one patient including FGFR3 Arginine 248 Cysteine mutation and on mutation at Serine 249 Cysteine.

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

The association between bladder cancer grad and FGFR3 mutations

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

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

<table>
<thead>
<tr>
<th>Mutation Grade</th>
<th>g.13515 del C</th>
<th>g.13515 del C</th>
<th>g.13529 ins A</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>12</td>
<td>8</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>G2</td>
<td>12</td>
<td>2</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>G3</td>
<td>12</td>
<td>2</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>12</td>
<td>3</td>
<td>51</td>
</tr>
</tbody>
</table>

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

In a study by (Khaldon et al., 2010) find a significant correlation (p < 0.001) between the stage of the tumor and FGFR3 mutations. However, in contrast to other studies, they could not find any correlation between the grade of the tumor and FGFR3 mutations. FGFR3 protein expression of moderate or high levels of protein in 49% of tumors but found no relationship to tumor grade or stage. Two other studies found a relationship between higher expression and lower tumor grade and stage (Gomez et al., 2005). Some of the studies have reported that low-stage and low-grade bladder tumors are associated with FGFR3 mutations (Van Tilborg et al., 2002; Hirao et al., 2005). The high frequency of FGFR3 mutations in pTa tumors, and their low frequency in pT1 and pT2–4 tumors are consistent with the model of bladder tumor (Lee and Droller, 2000; Knowles, 1999). FGFR3 mutations were detected at (21% pT1), (16% pT2 to pT4), twenty-seven from thirty-two (84% G1), Sixteen from
twenty-nine (55% G2) and five from twenty-one (7% G3) (Claude et al., 2001). FGFR3 protein expression of moderate or high levels of protein in 49% of tumors but found no relationship to tumor grade or stage. Other studies found a relationship between higher expression and lower tumor grade and stage (Gomez, et al., 2005). Other studies revealed that no mutations of the FGFR3 gene in bladder tumor were reported (Tomlinson et al., 2007; Arshad et al., 2010).

References


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