DNA SEQUENCING OF CAG REPEAT OF ANDROGEN RECEPTOR GENE OF PROSTATE CANCER PATIENTS IN THE SOUTH OF IRAQ

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ABSTRACT

The present study was designed to investigate some epidemiological and DNA sequencing study, in a sample of Thi-Qar, Basarh, Missan provinces south of Iraq. The sample included (32) patients with (32) healthy men as a control. The diagnosis was made by the consultant medical staff, which was based on a history inspection, and clinical examination. The epidemiological results were revealed that there were significant differences in the distribution of prostate cancer, where Basrah province recorded the highest percentage (75%) of prostate cancer in comparison with Thi-Qar in percent (15.6%) and (9.3%) in Missan. Our results revealed the highest number of patients filled in age (60-69) years and age (≥ 70) years, and low numbers in age group (50-59) years represented by (43.75, 40.62, and 15.62%, respectively). DNA sequencing examined (32) patients with prostate cancer and (32) controls to determined the relationship between prostate cancer and CAG repeat of androgen receptor gene, where shorter repeat recorded high percentage compared with controls (37.5% vs 3.1%), while longer CAG repeat of patients recorded low percentage compared with controls (62.5% vs 96.87%).

KEYWORD: Index Terms- prostate cancer, CAG repeat.

INTRODUCTION

The prostate is a gland found only in males. It is located in front of the rectum and below the urinary bladder. The size of the prostate varies with age. In younger men, it is about the size of a walnut, but it can be much larger in older men. The prostate's job is to make some of the fluid that protects and nourishes sperm cells in semen, making the semen more liquid.[1]
Prostate cancer remains one of the most common cancers afflicting men today. It is the third most common cancer in the world and the second cause of cancer death in men in Western countries.[2] Rates of detection of prostate cancers vary widely across the world, with Asia detecting less frequently than in Europe, and especially the United States.[3] Prostate cancer incidence and mortality rates are increasing in some Asian and European countries.[4] With lifestyle changes, the incidence of the disease has been increasing Arab population.[5] From 1991-2006, prostate cancer was ranked first among cancers in Qatari males over 65 years old.[6] In Kuwait, the incidence of prostate cancer rose to 12.3/100,000 men/year in 2004.[7] In 2003, prostate cancer was ranked as the fourth most diagnosed cancer in Tunisia.[8] In Lebanon, the incidence of prostate cancer was 21.5/100,000 men/year in 1998.[9] In Iraq, cancer of prostate is leading cause cancer in males accounting for 3.3% of the newly diagnosed cases.[10,11]

Several factors, including age, race, family history, hormone levels, and environmental influences are suspected to play a role in pathogenesis.[12]

Androgen receptor (AR) is a type of nuclear receptor, which is activated by binding dihydrotestosterone. AR is coded by gene located on the X chromosome at Xq11-12.[13] The main function of the AR is as a DNA-binding transcription factor, which regulates gene expression.[13,14] The AR contains two polymorphic repeats; a stretch of a variable number of the amino acid refer to CAG encoding for polyglutamine and the GGC repeats encoding for polyglycine.[15] Ethnical differences in the CAG repeat length are well known.[16] Shorter repeats in African populations, longer repeats in Asians, and intermediate lengths in Caucasians.[17] Studies showed a significant difference in transactivation between Androgen receptor s containing the shortest and the longest repeat, respectively, with a decreased activity for the longest repeat compared with the shortest. But neither the shorter nor the longer repeat differed in capability to activate the reporter gene.[18, 19] Short CAG repeat lengths have been proposed as increasing the androgen activity organized by AR, leading to increased sensitivity to cancer of prostate and benign prostatic hyperplasia. Shorter CAG repeat lengths are associated with advanced stage and high grade disease.[20]
MATERIAL AND METHOD
Blood samples were obtained by venepuncture, using a 5ml disposable syringe, from 100 suspected patients. 52 from them not suffering from prostate disease, 10 from them suffering from prostatitis and 6 from them suffering from benign prostatic hyperplasia. While, 32 were suffering from prostate cancer where contacts after surgical operation from the (Basrah oncology and hematology center) in Basrah, (Clinical oncology unit) in Thi-Qar, and (AL-Shefaa oncology center) in Misan. Also collected (32) blood samples from healthy men with the same age and state serve as control.

Genomic DNA Extraction
Genomic DNA from blood samples were extracted by using Genomic DNA Mini Kit (Blood/Cultured Cell) Geneaid. USA, and done according to company instruction.

Polymerase chain reaction (PCR)
Polymerase chain reaction assay was performed to detection of (CAG repeat of androgen receptor gene) from blood samples of prostate cancer patient and healthy samples. This method was done according to method described by.[21]

PCR Thermocycler Conditions
PCR thermocycler conditions were done by using conventional PCR thermocycler, as following table (1).

<table>
<thead>
<tr>
<th>PCR cycle</th>
<th>repeat</th>
<th>Temp.</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>95°C</td>
<td>5min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95°C</td>
<td>5sec.</td>
</tr>
<tr>
<td>Annealing</td>
<td>30</td>
<td>60°C</td>
<td>30sec.</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72°C</td>
<td>30sec.</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>72°C</td>
<td>7min</td>
</tr>
<tr>
<td>Hold</td>
<td>-</td>
<td>4°C</td>
<td>Forever</td>
</tr>
</tbody>
</table>

Primers of CAG repeat of androgen receptor gene
CAG repeat of androgen receptor gene primer that used in this study was according to protocol described by,[21], where provided from Bioneer company, Korea as following table (2).
Table (2): Primers and their sequence and size.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG repeats</td>
<td>5'-TCC AGA ATC TGT TCC AGA GCG TGC-3'</td>
<td>300bp</td>
</tr>
<tr>
<td></td>
<td>5'-GCT GTG AAG GTT GCT GTT CCT CAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

**Gel electrophoresis**

PCR products of CAG repeat was analyzed by loading in 1% Agarose, as following steps:

i. A 1% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100°C for 15 minutes, after that, left to cool 50°C.

ii. Then 3µ of ethidium bromide stain were added into agarose gel solution.

iii. Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (2000-100bp Ladder) in one well.

iv. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 80 volt and 60 AM for 1.5 hour.

v. PCR products (300bp) as specific for CAG repeat, were visualized by UV transmillumiation.

**DNA sequencing of CAG repeat of androgen receptor gene Method**

DNA sequencing method was performed for detection CAG repeat in PCR product of androgen receptor gene. The sequencing of the PCR product of androgen receptor gene, where the 300bp PCR product was purified from agarose gel by using (EZ-10 Spin Column DNA Gel Extraction Kit, Biobasic. Canada). As the following steps:

vi. The specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL microcentrifuge tube.

vii. A 400µl Binding Buffer II was added to gel fragment. Then, incubated at 60°C for 10 minutes and shaked until the agarose gel is completely dissolved.

viii. Add the above mixture to the EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000rpm for 2 minutes and discard the flow-through in the tube.

ix. A 750µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Then, solution discarded.

x. After that, the step 4 was repeated. Then, centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.
xi. The column was placed in a clean 1.5ml microcentrifuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and store at -20ºC.

xii. After that, the purified CAG repeat of androgen receptor gene PCR product samples were sent to Bioneer Company in Korea for performed the DNA sequencing by AB DNA sequencing system.

xiii. CAG repeat analysis was performed based on NCBI-Blast Alignment identification.

After that, the purified CAG repeat of androgen receptor gene PCR product samples were sent to Bioneer Company in Korea for performed the DNA sequencing by AB DNA sequencing system.

**Statistical Analysis**

Statistical analysis was performed with the Chi-square test. Categorical data were described as percentage; comparison done by using Chi-square test. P-value of ≤ 0.05 was used as the level of significance.

**RESULTS**

**Epidemiological study**

The epidemiology of prostate cancer in the south of Iraq (Basrah, Misan, Nassiriyah) was determined by analyzing the questioners filled up by the patients and control about their address, age and type of food consumed. State distribution of patient and control in south Iraq.

The distribution of patients with prostate cancer were mostly from Basrah in percent (75%), followed by Thi-Qar (15.6%) and Misan (9.3%) as shown in table (3).

**Table(3): State distribution of patients and controls.**

<table>
<thead>
<tr>
<th>State</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>Basrah</td>
<td>24</td>
<td>75</td>
</tr>
<tr>
<td>Thi-Qar</td>
<td>5</td>
<td>15.62</td>
</tr>
<tr>
<td>Missan</td>
<td>3</td>
<td>9.37</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>100</td>
</tr>
</tbody>
</table>
The P value was calculated between patient and control group. There are significant differences within the groups (P ≤ 0.01).

**Age distribution**

The number of patients was maximally significant (P ≤ 0.05) in age group (60-69) years (43.75%), and followed age group (≥ 70) years (40.6%) and then (15.6%) in age group 50-59, as summarize in table (4).

**Table (4): Age distribution of patients and controls.**

<table>
<thead>
<tr>
<th>Age group</th>
<th>Patients</th>
<th></th>
<th>Controls</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>50-59</td>
<td>5</td>
<td>15.62</td>
<td>5</td>
<td>15.62</td>
</tr>
<tr>
<td>60-69</td>
<td>14</td>
<td>43.75</td>
<td>14</td>
<td>43.75</td>
</tr>
<tr>
<td>≥ 70</td>
<td>13</td>
<td>40.62</td>
<td>13</td>
<td>40.62</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>100</td>
<td>32</td>
<td>100</td>
</tr>
</tbody>
</table>

The P value was calculated between patient and control group. Significant differences within the groups (P ≤ 0.05).

**DNA Sequencing of CAG repeat of androgen receptor gene result**

The present study found association between CAG repeat of AR gene and prostate cancer, where the shorter CAG repeat revealed highest association when compared with controls (37.5% vs 3.1%), while the longer CAG repeat of patients recorded lower percentage when compared with controls (62.5% vs 96.87%), as summarized in table (5). (We considered ≤ 18 CAG repeat as short and > 18 CAG repeat as long according to [21].

**Table (5): CAG repeat for patients and controls.**

<table>
<thead>
<tr>
<th>CAG repeat</th>
<th>Patients</th>
<th></th>
<th>Controls</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO.</td>
<td>%</td>
<td>NO.</td>
<td>%</td>
</tr>
<tr>
<td>Shorter</td>
<td>12</td>
<td>37.5</td>
<td>1</td>
<td>3.12</td>
</tr>
<tr>
<td>longer</td>
<td>20</td>
<td>62.5</td>
<td>31</td>
<td>96.87</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>100</td>
<td>32</td>
<td>100</td>
</tr>
</tbody>
</table>

The P value was calculated between patient and control group. Significant differences (P ≤ 0.01).
Figure (1): The sequence of an example patient sample. This patient had short (16) CAG trinucleotide repeat.

Figure (2): The sequence of an example control sample. This control had short (24) trinucleotide repeat.

DISCUSSION

Epidemiological study

The results of the present study showed a significant differences in the distribution of the prostate cancer patients in the south Iraq cities, and recorded increased of the prostate cancer in Basrah (75%) in comparison with Nassiriyah (15%), and Misan (9%) (Table 4.1), but that is not mean the percentage of the prostate cancer in these cities (Thi-Qar and Misan) were in low percent, these results may be due to the most cases were collected from Basrah\textsuperscript{[24]} cases, and these results may be due to the exposure to war weapon pollutant which may be promoted. This study revealed that the most patients with prostate cancer fall in group of age \( \geq 60 \) years (Table 4-2), this gives clear idea that there are a relationship between the disease and age, this confirmed by present study and by other previous studies of several authors\textsuperscript{[22]},...
who found that in united states more than (65%) of all prostate cancer are diagnosed in age men over the age of 65 and the average age diagnosis of prostate cancer is 69 years, after that age , the chance of developing prostate cancer becomes more common than any other cancer in men.[23], whose found that age is the most common risk factor with nearly 63% of prostate cancer cases occurring in men whose age was 65 and older, and in Iraq,[3], who found that in Baghdad city accounted (40%) of patients with Prostate cancer were in group of age (60 and above).

DNA sequencing of CAG repeat of androgen receptor gene
The variations of CAG repeat length associated with prostate cancer risk.[20] The length of the polymorphic CAG repeat sequence is inversely correlated with transcriptional activity of the androgen receptor.[24] Shorter CAG repeats have been associated with prostate cancer risk as well as an aggressive form of the disease. The present study revealed that shorter CAG repeat of the patients with prostate cancer was significantly high in patients compared with controls (37.5 vs 3.12%), as shown in table (4-12 and figures 4-10 and 11), we concluded that shorter CAG repeat can be consider as risk factor by compared with control, these results agreed with[25], found that a shorter CAG repeat sequence in the androgen receptor was associated with higher grade and advanced stage prostate cancer. Also agreed with[21], found that individuals with prostate cancer tend to have shorter CAG trinucleotide repeats. In the present study we concluded that shorter CAG repeat may be stimulate the activity of androgen receptor, which result in sensitive to cancer, this result agreed with[26], showed that decreased repeat length may result in an increase in consequent proliferative activity and somatic mutation rate.

REFERENCES


