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RESEARCH

Are GSTM1, GSTT1 and CAG Repeat Length of Androgen Receptor Gene Polymorphisms Associated with Risk of Prostate Cancer in Iranian Patients?

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Abstract We conducted this study to investigate whether CAG repeat length in androgen receptor gene and GSTM1 and GSTT1 polymorphisms influence prostate cancer risk in Iranian newly diagnosed cancer patients compared to age-matched BPH group and healthy individuals. DNA from 110 pathologically-confirmed prostate cancer patients, 99 age-matched men with Benign Prostatic Hyperplasia (BPH) and 100 healthy individuals were extracted and amplified by polymerase chain reaction (PCR). PCR products were examined by electrophoresis and sequencing. The mean number of CAG repeat in prostate cancer patients was significantly smaller than normal (19.9 vs 22.8; $p<$ 0.0001) and BPH groups (19.9 vs 21.9; $P<0.0001$) The mean difference between normal individuals and BPH group was also significant (21.9 vs. 22.8; $P=0.003$). Presence of GSTM1 null genotype were significantly higher in cancer and BPH group vs. normal individuals (both P values< 0.0001). there was not seen association between GSTT1 null or positive genotype with cancer risk, but analysis of GSTM1 null and GSTT1 positive in combination was statistically associated with Prostate cancer risk (OR=8.4, 95% CI 1.53–46.73). Our results showed that CAG repeat polymorphism in AR gene may act as a risk modifier and GSTM1 null genotypes also may be contributed to prostate cancer susceptibility in Iranian patients.

Keyword Prostate cancer · AR gene · CAG repeat · GSTM1 · GSTT1

Abbreviations

GST	Glutathione S-transferases
BPH	Benign Prostatic Hyperplasia
AR	Androgen Receptor
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
TRUS	Transrectal Ultrasound

Introduction

Prostate cancer is the most commonly diagnosed nonskin malignancy among men and the second leading cause of cancer death in western countries. Since the disease is more common among older men, its incidence is expected to increase as the population age elevated [1]. The molecular genetics of prostate cancer is poorly understood. Its heterogenous nature suggests that predisposition to prostate cancer may involve multiple genes and variable phenotypic expression. Heterogeneity also increases markedly with progression from benign through prostatic intraepithelial neoplasia (PIN) to localized prostate cancer and metastasis [2].

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The growth, differentiation, and proliferation of prostate cells are regulated by androgens. The biological effects of androgen is mediated through binding to intracellular androgen receptors (AR), which in turn regulate the transcription of target genes with the assistance of transcriptional coactivator [3].

The human AR is encoded by a single copy gene which is located at Xq11.2-q12, and has more than 90 kb length. The coding region of AR contains eight exons. The first exon encods a large amino-terminal transactivating domain, and contains several polymorphic repeats. The most variable is polymorphic CAG repeats, which encodes a polyglutamin chain in the transactivation region of the AR [4]. The polymorphic CAG trinucleotide repeat is inversely correlated with transactivation of AR. Therefore, longer polyglutamin tract hinders transactivation activity of AR, whereas, shorter chain causes more activation.

Conflicting evidence exists as to whether shorter alleles of CAG repeats confer a greater risk of prostate cancer. In several studies, it has been reported that shorter CAG repeat length is associated with an increased risk of prostate cancer and benign prostate hyperplasia. However, some of studies provide evidence that CAG repeat in AR gene has no association with the risk of prostate cancer [5]. Various results about shorter alleles of CAG repeats in relation to prostate cancer and BPH in different ethnic groups (Austrian, Caucasian, African and American), and also there are a few reports from Asia on AR polymorphism with no data from Iran.

There are other genetic polymorphisms that may associate with prostate cancer such as Glutathione S-transferases (GSTs). One of the phase II metabolic enzymes are GSTs which are mostly involved in activating and inactivating oxidative metabolites of carcinogenic compounds associated with causing prostate cancer [6–8]. In addition they also play a very significant role in regulating the activation of another enzymes for DNA repair and some other cellular functions [6]. Eight loci are seem to be responsible for coding GST enzymes: α (GSTA), μ (GSTM), θ (GSTT), π (GSTP), σ (GSTS), κ (GSTK), o (GSTO), and τ (GSTZ), each of which include some isoforms [9, 10]. Most studies focused on GSTM1 and GSTT1 polymorphisms than others [11]. Different frequencies of the GSTM1 and GSTT1 homozygous deletions “the null genotype” have been reported between ethnic groups [12].

In this study we investigated whether the variable size and shorter repeats of trinucleotide could alter AR function and result in developing prostate cancer in Iranian newly diagnosed prostate cancer patients compared to age-matched BPH group and healthy individuals. Also we determined the frequency of the GSTM1 and GSTT1 null genotype and their association with the risk of prostate cancer.

Materials and Methods

Subjects

The study group comprised of 110 previously untreated prostate cancer patients with known histological information, 99 age-matched BPH untreated and 100 normal individuals. Indication for prostate cancer and BPH was elevated serum level of prostate specific antigen (PSA) and further diagnosis was made by transrectal ultrasound-guided biopsies (TRUS). Pathological examination confirmed the cancerous cells or benign hyperplasia. For all patients, relevant clinical and pathological data were collected. Written informed consent was obtained from all participants in this research. Approval was obtained through the Investigation review Board at the Tehran University of Medical Sciences. This study was also approved by the ethics and clinical studies committee of Tehran University of Medical Sciences.

DNA Extraction

Blood and biopsy samples were collected from patients and normal individuals. DNA was extracted from the peripheral blood by salting-out method and using proteinase K.

Molecular Analyses of CAG Repeat Length

The CAG length was determined by analyzing the size of PCR product containing the polymorphic microsatellite. Nested polymerase chain reaction (PCR) was carried out using extracted DNA in a series of two rounds of amplification. We designed two sets of oligonucleotides from sequence of exon 1, the first as a pair of external primers: forward, 5′-TCCAAGACCTACCGAGGAGC-3′; reverse, 5′-AGGGCCGACTGCGGCTGT-3′. The second set, nested primers, designed specifically for the CAG repeats: forward, 5′-TCCAGAATCTGTTCCAGA GCGTGC-3′; reverse, 5′-GCTGTGAAGGTTGCTGTTC CTC-3′. Total volume of 25 μl reaction mixture contained about 100 ng DNA, PCR buffer, 1.5 mM MgCl2, 200 μmol deoxynucleotide triphosphate, 10 pmol of each primer and 2 U SmarTaq polymerase (Cinnagen, Iran). PCR condition that optimized for external primers was: initial denaturation 94°C for 5 min, 30 cycles each consisting of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C followed by a final extension at 72°C for 7 min. The second PCR for CAG repeat initiated with denaturation at 94°C for 5 min and then 30 PCR cycles consisting of 1 min at 94°C, 1 min at 63°C, 1 min at 72°C, and with a final extension 7 min at 72°C. Because the PCR procedure is prone to contamination, a negative control was always included in each set of PCR reaction, and also a positive control was used for checking the PCR reaction.

PCR products were examined by electrophoresis on 1% agarose gel, and analyzed by running on 10% polyacrylamid gel. For identification of exact size of CAG repeats, products were analyzed by sequencing (Applied Biosystems 3730/3730*xl*).

Molecular Analysis of GSTM1 and GSTT1 Polymorphisms

A multiplex PCR assays was performed on extracted DNA in order to investigate GSTT1 and GSTM1 polymorphisms, simultaneously, β globulin gene was used as an internal positive control using these primer sets FWD 5′ CAACTT CATCCACGTTCACC 3′ and REV 5′ GAAGAGCCAAG GACAGGTAC 3′. Primer set applied for identification of GSTM1 and GSTT1 were FWD 5′ CAACTTCATCCA CGTTCACC 3′, REV 5′ GAAGAGCCAAGG ACA GGTAC 3′ and FWD 5′ TTCCTTACTGGTCCTCA CATCTC 3′ and REV 5′ TCACCGGATCATGGCCAGCA 3′ respectively. Total volume of 25 μl reaction mixture contained about 100 ng DNA, PCR buffer, 1.5 mM MgCl2, 200 μmol deoxynucleotide triphosphate, 10 pmol of each primer and 2 U SmarTaq polymerase (Cinnagen, Iran). PCR condition for this multiplex PCR assays was: initial denaturation 94°C for 5 min, 35 cycles each consisting of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C followed by a final extension at 72°C for 7 min. Amplicons were analyzed by electrophoresis on a 2% Agarose gel followed by ethidium bromide staining. GSTM1 null genotype was defined as absence of 215-bp band after electrophoresis, GSTT1 null genotype established by absence of 459-bp fragment, and a positive β globulin (positive internal control) was identified with a 273 b-bp fragment.

Statistical Analysis

Analysis of data was performed using the SPSS for windows version 15 (SPSS Inc., Chicago, IL, USA). Mean number of CAG, PSA level, prostate weight and Gleason score were calculated. *T*-test and Tukey test were used to compare these parameters between different groups.). Chi-square and Fisher's exact test was used to compare GSTM1 and GSTT1 genotype among prostate cancer patients, BPH and normal groups. The number of CAG repeats was examined as linear and categorized variables. The odd ratios (OR) corresponding to 95% confidence interval (95% CI) were calculated. *P* values≤0.05 are considered statistically significant.

Results

The mean age of prostate cancer cases, BPH and normal groups were 69.5, 64.5 and 60.4 years, respectively. Mean prostate weight of prostate cancer and BPH groups were 48.1(42–53) and 64.2(57–70), respectively. The mean PSA level was 21.2 ng/ml in cancer cases, comparing to 12.7 ng/ml in BPH group. The difference was significant (*t*-test, $P=0.004$). The mean prostate weight in cancer patients was significantly lower than the other group ($P<0.001$). Distribution of CAG repeat length in prostate cancer, BPH and normal prostate tissues are shown in Fig. 1. The CAG repeat number ranged from 15 to 24 in cancer cases, 19–26 in BPH group and 13–26 in healthy individuals (Table 1). The mean CAG repeat length in cancer was significantly smaller than normal (19.9 vs. 22.8) (*t*-test, $P<0.0001$) and in comparison to mean CAG repeat in BPH group (19.9 vs. 21.9), there was a significant difference with $P<0.0001$. The difference observed between normal individuals and BPH group (22.8 vs 21.9) was significant ($P=0.004$). We also used Tukey test to compare three groups with each other. Significant differences were observed ($P<0.0001$), which confirm results obtained from *t*-test. Analysis of the repeat length with respect to patients' age showed that the age at diagnosis was not related to size of this polymorphism. The CAG repeat length was used to dichotomize the study subjects, men with short alleles [13–20] and long alleles [21–26] (Tables 2, 3). Men with CAG repeat length ≤21 had ten fold increases risk for cancer than those with length >21 (OR=10.1, CI 95%; 4.38–23.3).

Cancer cases had higher frequency for CAG repeat length of ≤21 in comparison to BPH group and normal individuals (60.9%, 16.1 and 15%, respectively). The

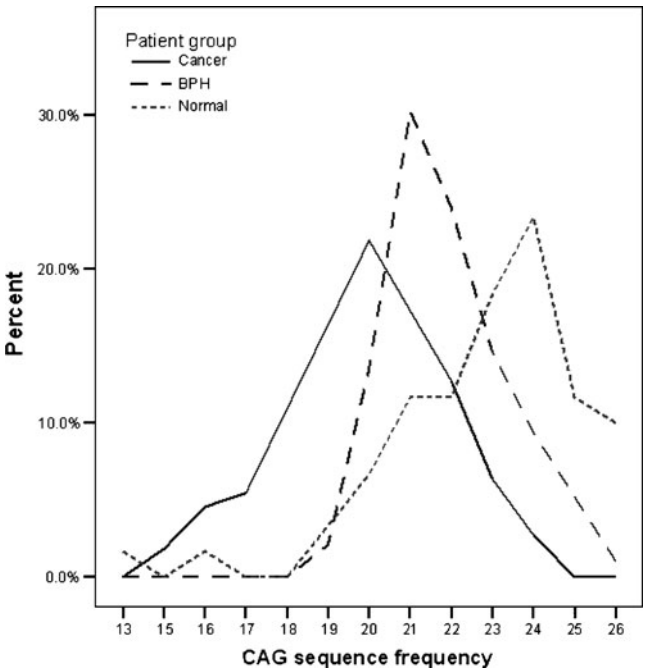

Fig. 1 CAG repeat length frequency in cancer patients, BPH and normal groups

Table 1 CAG repeat Length in subject groups

	Characteristic	Prostate cancer patients [*n*]	BPH group [*n*]	Normal Individuals[*n*]
CAG repeat				
	Mean (95%CI)	19.9[19.53–20.29]	21.9[21.60–2.20]	22.8[22.17–2.43]
	Minimum	15	19	13
	Maximum	24	26	26

association of dichotomized AR repeats with Gleason score in 110 prostate cancer patients was analyzed and shown in Table 4.

Prevalence and Odds Ratios (OR) of GSTT1 and GSTM1 genotypes; among Prostate Cancer Cases and BPH group are shown in Table 3 and among Cancer patients and normal individuals are displayed in Table 2. Frequency of GSTM1 null genotype was significantly higher in cancer and BPH groups than normal individuals (Both P values<0.0001). There was an increased risk of prostate cancer in patients with GSTM1 null genotype in comparison to normal group (OR=7.107, 95% CI 3.180–15.881). While we did not observe a significant difference of GSTM1 null genotype between cancer and BPH groups. GSTT1 null genotype frequency among cancer patients, BPH patients and normal individuals was 34.6%, 37.3 and 47% respectively. We did not reach a significant difference between these three groups for GSTT1 null genotype. GSTT1 and GSTM1 genotypes had not a significant relation with either of PSA level, prostate weight or Gleason score of patients.

We have also analyzed the effects of CAG repeat ≤21 with either of GSTM1 or GSTT1 genotypes in combination. We have found no association between PSA level, patients' age and gleason score and combined genotypes in relation to prostate cancer. There was not a higher risk of prostate cancer for combined genotypes of CAG and GSTM1 or GSTT1, but the analysis of GSTM1 and GSTT1 genotype in combination (between cancer and normal groups) revealed 8.4 times increased prostate cancer risk for GSTM1 Null and GSTT1 positive(OR=8.4, 95% CI 1.53–46.73).

Discussion

Androgen action is mediated through the androgen receptor (AR), which has potential roles in mediating androgen hormone function. The AR acts as a ligand dependent transcriptional regulator, which is important in controlling prostate growth and apoptosis. Androgen action and the functional status of AR are great mediators for prostate cancer progression. Variation in transcriptional activity of AR related to polymorphic CAG repeats, located in exon 1, may lead to prostate carcinogenesis. Since increased androgenic activity has been linked to prostate cancer, it has been proposed that men with shorter repeats will be at higher risk for prostate cancer [13].

This investigation confirmed the hypothesis that a shorter CAG repeat length is associated with an increased risk of clinically significant prostate cancer. To our knowledge, this study is the first report of CAG repeat length variation in prostate cancer patients, BPH group, and normal individuals in Iran. We found that individual with CAG repeat ≤21 have ten times greater risk for cancer than those with >21 repeats, which is more than Hispanic and Indian populations. Hispanic men with a repeat length of 18 or less and Indian men with ≤22 showed an approximately 3-fold increased risk of prostate cancer [3, 14]. However, a 3.7 risk has also been reported on comparison of shorter CAG repeats (≤17 vs. >17) to longer repeat [15]. We also analyzed the prostate cancer risk by grouping the CAG repeats in dichotomous according to BPH and normal. There was an increased risk of prostate cancer with shorter alleles as compare to longer CAG repeat dichotomous. As shown in Fig. 1 the most frequent allele in cancer patients

Table 2 CAG repeat, GSTM1 and GSTT1 distribution and risk assessment of prostate cancer comparing to normal

Characteristic		Prostate cancer [*n* (%)]	Normal	*P* value	OR	CI
N of patients		110	100			
Number of CAG repeats	>21	43(39.1)	52(52)	<0.0001	10.1	4.3–23.3
	≤21	67(60.9)	15(15)			
GSTT1		72(65.4)	53(53)			
null		38(34.6)	47(47)	0.76	0.58	0.317–1.061
GSTM1		60(54.5)	90(90)			
null		50(45.5)	10(90)	<0.0001	7.107	3.180–15.881

Table 3 CAG repeat, GSTM1 and GSTT1 distribution and risk assessment of prostate cancer comparing to BPH

Characteristic		Prostate cancer	BPH	*P* value	OR	CI
N of patients		110	99			
Number of CAG repeats	>21	43(39.1)	83(83.9)	<0.0001	8.4	4.3–16.4
	≤21	67(60.9)	16(16.1)			
GSTT1		72(65.4)	62(62.7)			
null		38(34.6)	37(37.3)	0.662	0.87	0.458–1.641
GSTM1		60(54.5)	52(52.5)			
null		50(45.5)	47(47.5)	0.705	0.888	0.481–1.640

was 20 repeats, whereas, in BPH and normal groups were 21 and 24 repeats, respectively.

The AR CAG allele has well-established population differences [3, 5, 13, 14]. The most frequent allele in Chinese prostate cancer patients was 22, but, the common allele in Indians, Australian and white American patients with the cancer was 21 repeats. It has been reported that in Austrian Caucasian men with BPH, 21 repeats allele was the most frequent allele, which is similar to our results.

It was published that short CAG repeats could predispose to more aggressive form of prostate cancer. However, we did not find relation between this polymorphism and Gleason score, despite of 69.8% cancer cases with Gleason score ≥7 in this study. There was no association between the polymorphism and age as well as PSA level and Gleason score.

Variation in repeat alleles range was observed in different population. It may speculate that genetic susceptibility is more important than age in incidence of prostate tumor. The AR gene transcriptional activation is influenced not only by polymorphisms in this gene but also by other factors including tissue levels of dihydrotestosterone, estradiol, insulin-like growth factor and AR coactivator [4]. Apart from genetic factors, environmental, nutritional, hormonal may be the causes of prostate cancer. These factors may also affect prostate cancer risk by mediating transcriptional activities.

A multi gene family expresses different GSTs and there have been reported extensive genetic polymorphisms in these genes. In most studies GSTM1 and GSTT1 polymorphism and cancer have compared the homozygous deletion genotype (null) with the heterozygous or homozygous presence genotype [16]. Null genotype frequency in GSTM1 polymorphism ranges from 22.7% to 54.2% in Caucasians, 42.6% to 54.5% in Asians, 27.1% to 46.7% in African–Americans. Meanwhile the GSTT1 null genotype were 13%–58.7% in Caucasians, 26.7% in African–Americans, and 41.9%–48.3% in Asians [16].

The frequency of the GSTM1 null genotype was slightly lower in prostate cancer patients (45.5%) as compared with BPH group (47.5%) while in other studies, various results were achieved. Gsur et al. have reported 45.2% and 48.8% of GSTM1 null genotype in prostate-cancer patients and BPH control group respectively, which as in our study, the relation was not statistically significant [17]. Some investigations have not found a statistical relationship with GSTM1 null genotype and prostate cancer development in comparison to their BPH group [18, 19] but others showed significant difference [20]. Some other studies have used normal (non BPH) controls and showed significant associ-

Table 4 Association of dichotomous of CAG repeats with Gleason score, age and PSA in patients with prostate cancer

	CAG repeat length			
	13–20 *n* (%)	21–26 *n* (%)	OR (CI 95%)	*P* value
Total prostate cancer	67(60.9)	43(39.1)		
Gleason >7	41(70.7)	26(68.4)		
Gleason ≤7	17(29.3)	12(31.6)	0.89(0.37–2.18)	0.56
Age				
>69	31(46.3)	24(55.8)		
≤69	36(53.7)	19(44.2)	1.47(0.68–3.17)	0.95
PSA				
>10	42(62.7)	24(55.8)		
≤10	25(37.3)	19(44.2)	0.75(0.34–1.63)	0.51

ation in null alleles of the GSTM1 and higher risk for prostate cancer [7, 21–23]**.** we also used normal age-matched individuals as control. Frequency of GSTM1 null genotype was significantly higher in cancer patients (45.5% vs. 10%, *P* values<0.0001) and is associated with seven fold increased cancer risk (OR=7.107, 95% CI 3.180–15.881); However, there are contradictory reports [11, 24].

GSTT1 null genotype found in 34.6% of patients which did not differ significantly from those in normal and BPH control groups, which is similar to Murata et al. findings [18]. Mittal et al. demonstrated that the GSTT1 null genotype was significantly higher in cancer patients in comparison to BPH group [25]. We have not seen association between GSTT1 null or positive genotype with cancer risk in comparison to normal control. But when the GSTM1 null and GSTT1 positive polymorphisms were analyzed in combination, it was statistically associated with prostate cancer risk (OR=8.4, 95% CI 1.53–46.73). Some studies have reported combined analysis and association of GSTM1 and GSTT1 polymorphism [22, 26, 27]. It can be related to gene—gene interaction which Srivastava in 2005 suggested that it may be contributed to a tendency for developing prostate cancer. It should be mentioned that combination analysis of between AR and GSTM1 or GSTT1 have revealed no association.

Molecular epidemiological studies have now provided evidence that both genetic and environmental factors modulate individual's susceptibility to cancer. Since GSTs have a significant role in regulation of enzymes activation for DNA repair and some other cellular functions, probably GSTM1 gene product is one of regulatory factor that specifically effect on enzymes that control DNA repair because homozygote deletion (null genotype) is associated with prostate cancer. As GSTs role is expected to be protective [8, 28], null genotype of GSTM1 may be increases susceptibility to cancer.

According to our results CAG repeat in AR gene with shorter allele and GSTM1 null genotype are strongly predisposing risk factors for prostate cancer. Even so, the preference of our study to others is using both BPH group and normal individuals as control. Further studies with larger number of healthy controls and patients are possibly needed to evaluate the effect of AR, GSTM1 and GSTT1 polymorphisms with other coactivators that were mentioned.

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