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Evaluation of the effects of androgen receptor gene trinucleotide repeats and prostate-specific antigen gene polymorphisms on prostate cancer

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ABSTRACT. The number of trinucleotide repeats [CAG (coding for polyglutamine), GGC (coding for polyglycine)] in the first exon of the androgen receptor (AR) gene and prostate-specific antigen (PSA) gene androgen response element I A/G polymorphism are both related to prostate cancer prognosis. We investigated whether these genomic changes occur in the AR and PSA genes, which are usually found in individuals with prostate cancer, of Turkish patients and to find out their distribution in the population. We used PCR and PCR-RFLP assays for AR and PSA genes, respectively, to detect molecular changes in 44 prostate cancer patients. Our findings indicate that individuals with prostate cancer tend to have around 18 CAG trinucleotide repeats. We observed significant differences between 22 controls, 33 benign prostate hyperplasia (BPH) patients and 44 adenocarcinoma patients for long CAG repeats. However, we did not find any significant differences in GGC repeats between controls, BPH and adenocarcinoma patients (P = 0.408). We also did not observe significant

differences in the PSA A/G polymorphism frequency between controls, BPH and adenocarcinoma patients (P = 0.483). In conclusion, CAG and GGC repeats in the AR and PSA gene polymorphisms may be associated with prostate cancer risk and BPH in the Turkish population.

Key words: Prostate cancer; Androgen receptor (AR) gene; Prostate-specific antigen (PSA) gene; Trinucleotide repeats

INTRODUCTION

In men, prostate cancer ranks second to lung cancer in cancers that are likely to result in death. Recent studies have shown that prostate cancer has a genetic basis, which could be important for inheritance of the cancer to male offspring. These genes are the androgen receptor (AR) gene, prostate-specific antigen (PSA) gene, 5 α-reductase type II (SRD5A2) gene, cytochrome P450c17α (CYP17) gene, cytochrome P4503A4 (CYP3A4) gene, and elaC homolog 2 (ELAC2) genes (Gsur et al., 2002). Mutations in the AR and PSA genes are thought to be responsible for the development of prostate cancer (Stanford et al., 1997; Tavtigian et al., 2001; Gsur et al., 2002, 2004; Nenonen et al., 2010).

The development and progression of prostate cancer is affected by androgens. The actions of androgens are mediated via an AR, which is a ligand-dependent transcription factor. The single-copy human AR gene is located at the Xq11.2-q12 chromosome X (Lubahn et al., 1989; Monroe et al., 1995; Xu et al., 1998; Lange et al., 1999) and its activity is regulated by testosterone and dihydrotestosterone. In prostate cancer, AR may show similar activity to the members of other steroid receptor families (Evans, 1988). The DNA-binding domain in the first exon of AR has a zinc finger domain that may bind to androgen response element(s) (ARE) at the N terminal, and to androgen at the C terminal. This exon contains several polymorphic repetitions of which CAG (coding for polyglutamine) and GGC (coding for polyglycine) show the most variability. Normally there are 8-35 CAG repeats, the average being 20. These repeats form a polyglutamine chain on the AR protein that can bind to DNA to regulate transcription. An increase in the number of CAG repeats may interfere with the transcription of the AR gene. However, transcriptional activity may increase when repeats are high. Therefore, shorter repeats may comprise higher risk for prostate cancer (Irvine et al., 1995; Zeegers et al., 2004; Mishra et al., 2005; Krishnaswamy et al., 2006; Rodríguez-González et al., 2009).

The effect of the variation in the length of the GGC tract on AR activity is unclear, but it has been thought that the GGC trinucleotide repeats range from 10 to 30 the transcriptional activity of the AR gene (Gao et al., 1996; Zeegers et al., 2004; Mishra et al., 2005; Krishnaswamy et al., 2006). However, to date, no relationship has been established between the length of the polyglycine region and infertility (Tut et al., 1997). Epidemiological studies have shown that a decrease in the number of repeats in both sequences constitutes a major risk for the occurrence of prostate cancer (Stanford et al., 1997; Hakimi et al., 1997; Platz et al., 1998; Sartor et al., 1999). Indeed, prostatectomy has been recommended for individuals, who have a decreased number of repeats, as the prostate cancer risk is considerably higher in this individual (Hardy et al., 1996; Bratt et al., 1999).

The PSA gene, a member (hK3) of the human kallikrein (hK) gene family, is located at the 19q13.41 chromosome 19 (Klobeck et al., 1989; Lilja, 2003). It encodes a glycoprotein

containing 240 amino acids, also known as serine protease (33 kDa). Since PSA secretion is regulated by androgens via AR-dependent pathways, an increase in the amount of androgen in turn induces PSA secretion from the prostate epithelium (Medeiros et al., 2002; Rao et al., 2003). Therefore, ARE mutations interfering with the PSA regulation lead to an increase in PSA secretion. An increase in PSA secretion will eventually cause prostate cells to develop into tumor tissue (Xu et al., 1998; Lange et al., 1999).

There are three different ARE sites in the promoter region of the PSA gene, which are known to regulate PSA gene transcriptional activity. These AREs are ARE I, ARE II and ARE III (Rao et al., 2003). Androgen response element I contains two allele variants (AGAACAnnnAGT**G**CT and AGAACAnnnAGT**A**CT). The AR binds these different alleles with different affinities. Therefore, these variations in affinities are important in the development of prostate cancer risk (Xue et al., 2000, 2001). In a study carried out on males with prostate cancer in Portugal, ratios of AA, AG and GG genotypes were determined as 43, 41 and 16%, respectively (Medeiros et al., 2002). The same genotypes were detected as 5, 31 and 64% in healthy Japanese males (Wang et al., 2003). Another study carried out in China in randomized samples of the same genotypes was determined as 5, 26 and 69% (Liu et al., 2003). In addition to that, the GG genotype is reported to have a higher risk of prostate cancer especially among younger men (Binnie et al., 2005). The purpose of this study was to examine the relationship between benign prostate hyperplasia (BPH), malignant prostate cancer (adenocarcinoma) and polymorphisms in two genes involved in the androgen pathway, the CAG and GGC trinucleotide repeat length in the AR gene and the A/G polymorphism at the ARE I region of the PSA gene.

MATERIAL AND METHODS

This study was performed on 33 patients with BPH (61.06 ± 11.263 mean age), 44 patients with malignant (adenocarcinoma) cancer (64.24 ± 8.89 mean age) and 22 controls (58.14 ± 9.671 mean age) with no known cancer. Patients were referred to the Urology Department at Çukurova University, Adana, Turkey. Blood (5 mL) was obtained from each patient. Genomic DNA was isolated employing the standard salting-out DNA isolation method (Sambrook et al., 1989) and then amplified with gene specific primers. For the CAG repeats, the polymerase chain reaction (PCR) products of the AR gene were obtained using a mixture containing 10 pmol from each primer: 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3' (forward) and 5'-GCT GTG AAG GTT GCT GTT CCT CAT-3' (reverse) (Xu et al., 1998), 2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Taq polymerase, and 100-500 ng DNA sample. After the initial denaturation of the reaction mixture at 95°C for 3 min, amplification was achieved by 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min (Xu et al., 1998). For the GGC repeats, the PCR products of the AR gene were obtained using a mixture containing 10 pmol from each primer: 5'-TCC TGG CAC ACT CTC TTC AC-3' (forward) and 5'-GCC AGG GTA CCA CAC ATC AGG T-3' (reverse) (Xu et al., 1998), 2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Taq polymerase, and 100-500 ng DNA sample. After initial denaturation of the reaction mixture at 95°C for 3 min, amplification was achieved by 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min (Xu et al., 1998). The PCR products were loaded onto 10% polyacrylamide gels and electrophoresed at 100 V for 90 min, stained in EtBr for 5 min, visualized by using a UviTech Gel documentation system and then evaluated.

The ARE in the promoter region of the PSA gene was amplified in a PCR mixture con-

taining 10 pmol from each primer: 5'-TTG TAT GAA GAA TCG GGG ATC GT-3' (forward) and 5'-TCC CCC AGG AGC CCT ATA AAA-3' (reverse), 2 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Taq polymerase, and 100-500 ng DNA sample for each reaction. After the initial denaturation of the reaction mixture at 95°C for 3 min, amplification was achieved by 30 cycles at 95°C for 30 s, 59°C for 30 s and 72°C for 30 s, and a final extension at 72°C for 10 min (Xue et al., 2000). The A/G polymorphism at the ARE I (AGA ACA GCA AGT *G*CT AGC) region was detected by the RFLP (restriction fragment length polymorphism) method. Restriction digestion of the PCR products was performed by using the *Nhe*I enzyme (Xue et al., 2000). After digestion, the original PCR products and restriction digestion products were loaded onto 10% polyacrylamide gels and electrophoresed at 100 V for 90 min, stained in EtBr for 5 min, visualized by using a UviTech Gel documentation system and then evaluated. As a result of our evaluation we observed a 300-bp band for homozygous AA, 300- and 150-bp bands for heterozygous AG and a 150-bp band for homozygous GG.

The analysis of the data was performed by the SPSS software (version 11.5). The Pearson chi-square test was used to compare ratios and $P < 0.05$ was accepted as statistically significant. Additionally, we estimated the area under the curve (AUC) and corresponding 95% confidence interval (CI) for the association between each repeat length and prostate cancer. In our analysis, we used 18 cut-off points for the CAG repeats but could not determine the cut-off points for analyzing the GGC repeats. We therefore analyzed the GGC repeats with the Kruskal-Wallis test.

RESULTS

Our study comprises prostate cancer patients from the Cukurova region, Turkey. In the present study, the number of trinucleotide repeats in the first exon of the AR gene (CAG and GGC repeats) and PSA gene polymorphisms were investigated. This study was performed on 33 BPH patients (61.06 ± 11.263 mean age), 44 adenocarcinoma patients (64.24 ± 8.689 mean age) and 22 healthy controls (58.14 ± 9.671 mean age). When analyzing the CAG repeats, we used 18 as a cut-off point (AUC = 0.659 ± 0.055; 95%CI = 0.552-0.766; P = 0.007) (Table 1). The AR CAG repeat sizes were categorized as "short" (≤18 CAG repeats) or "long" (>18 CAG repeats).

Table 1. Coordinates of the curve.

Positive if greater than or equal to	Sensitivity	Specificity
8.00	1.000	1.000
9.50	0.977	0.945
11.00	0.977	0.927
12.50	0.977	0.855
13.50	0.977	0.836
14.50	0.932	0.800
15.50	0.909	0.727
16.50	0.773	0.564
17.50	0.727	0.473
18.50	0.591	0.400
19.50	0.523	0.400
20.50	0.432	0.218
21.50	0.318	0.164
22.50	0.205	0.091
23.50	0.114	0.055
24.50	0.000	0.036
26.00	0.000	0.000

Frequencies of the AR CAG repeats in controls, BPH and adenocarcinoma patients are shown in Table 2. The difference between controls, BPH and adenocarcinoma patients was statistically significant (P = 0.03; P < 0.05). We also observed that the frequencies of the CAG repeats increase as the adenocarcinoma develops. The control, BPH and adenocarcinoma groups have different long CAG repeat frequencies, which are 14.6, 31.2 and 54.2%, respectively.

Table 2. Distribution of the CAG repeats between controls, BPH and adenocarcinoma.

Genotypes	Control (N = 22)	BPH (N = 33)	Adenocarcinoma (N = 44)	Total (N = 99)	P value
Short repeat	15	18	18	51	0.03
	29.4%	35.3%	35.3%	100%	<0.05
Long repeat	7	15	26	48	
	14.6%	31.2%	54.2%	100%	

BPH = benign prostate hyperplasia.

During GGC repeat analyses, we could not determine a specific cut-off point (AUC = 0.573 ± 0.087; 95%CI = 0.403-0.743; P = 0.386). Therefore, the mean of the GGC repeats of the groups has been determined with the Kruskal-Wallis test. Control, BPH and adenocarcinoma groups have means of 28.75, 21.31 and 27.39%, respectively. We did not observe any difference between the controls, BPH and adenocarcinoma patients (P = 0.408; P > 0.05).

In the present study, we examined an A/G polymorphism in the promoter region of the PSA gene (Table 3). We found the percentages of AA, AG and GG variants to be as follows: the AA variant in controls 14.6%, in BPH 42.7% and in adenocarcinoma patients 42.7%; the AG variant in controls 25.7%, in BPH 17.1% and in adenocarcinoma patients 57.2%; the GG variant in controls 11.1%, in BPH 22.2% and in adenocarcinoma patients 66.7%. However, we could not establish a statistically significant correlation for the A/G polymorphism in the PSA gene between controls, BPHs and adenocarcinoma patients (P = 0.483; P > 0.05).

Table 3. Distribution of PSA A/G polymorphism between controls, BPH and adenocarcinomas.

PSA A/G genotypes	Control (N = 11)	BPH (N = 11)	Adenocarcinoma (N = 29)	Total (N = 51)	P value
AA	1	3	3	7	0.483
	14.6%	42.7%	42.7%	100%	>0.05
AG	9	6	20	35	
	25.7%	17.1%	57.2%	100%	
GG	1	2	6	9	
	11.1%	22.2%	66.7%	100%	

BPH = benign prostate hyperplasia.

When PSA genotypes and CAG repeats were assessed together, it was found that the short CAG repeat frequencies of the AA genotype was 16.7%, AG genotype was 58.3% and GG genotype was 25.0%. It was also found that the long CAG repeat frequencies of the AA genotype was 12.5%, AG genotype was 83.3% and GG genotype was 4.2% (Table 4). Nevertheless, we did not observe any statistically significant differences between CAG repeats and PSA A/G polymorphisms (P = 0.09; P > 0.05).

Table 4. Distribution of PSA A/G polymorphism between short and long CAG repeats.

PSA A/G genotypes	Short repeat (N = 24)	Long repeat (N = 24)	P value
AA	4	3	0.09
	16.7%	12.5%	>0.05
AG	14	20	
	58.3%	83.3%	
GG	6	1	
	25.0%	4.2%	
Total	24	24	
	100%	100%	

DISCUSSION

The AR gene contains two polymorphic trinucleotide repeats within the transactivation domain of the first exon, which are the CAG and the GGC repeats. The length of the CAG repeat is inversely related to AR transcriptional activity. Shorter CAG repeats have been associated with prostate cancer risk as well as an aggressive form of the disease. The relationship between the length of GGC repeats and prostate cancer risk, however, is not clearly understood (Irvine et al., 1995; Zeegers et al., 2004; Mishra et al., 2005; Krishnaswamy et al., 2006).

Androgens are critical for the development and progression of prostate cancer. It has been suggested by Ross et al. (1999) that variation in transcriptional activity in the AR is related to polymorphic CAG repeats affecting prostate carcinogenesis. Short CAG repeats may be associated with a higher level of transactivation, thus increasing the risk of prostate cancer (Ross et al., 1999). The number of CAG repeats associated with prostate cancer as a continuous variable was 0.97 (95%CI = 0.95-1.03), suggesting a 3% decrease in prostate cancer risk for each additional CAG repeat (Stanford et al., 1997) but this result was not statistically significant. Gsur et al. (2002) reported that these data provide no evidence for an association between prostate cancer and CAG repeat length.

In the present study, we observed statistically significant differences between controls, BPH and adenocarcinoma for long CAG repeats ($P = 0.03$; $P < 0.05$). Our results may imply that longer CAG repeats might be associated with a higher level of receptor transactivation, since the number of CAG repeats increases in the BPH and adenocarcinoma patients compared to controls with the highest increase in adenocarcinoma patients.

Lack of a uniform model of analysis also makes these studies difficult to compare. Giovannucci et al. (1997, 1999) examined CAG repeat length as a continuous variable and also compared men with ≤18 repeats, and men with ≥26 repeats. Irvine et al. (1995) and Stanford et al. (1997) used the median number of 22 to divide their population for analysis. Lange et al. (1999) declared that it is unclear whether these studies examined multiple cut-off points and were appropriately corrected for multiple testing. In our study, we determined a cut-off point of ≤18 as important for evaluating the data. The association of the short CAG repeats and prostate cancer incidence, as well as the variety of different models, made it possible to interpret the data.

There are similar uncertain data for the GGC repeats and prostate cancer, with some (Stanford et al., 1997; Hakimi et al., 1997; Platz et al., 1998), but not all (Correa-Cerro et al., 1999) studies reporting an association, while other studies suggest that longer AR GGC repeats may increase risk among men with a positive family history of prostate cancer. Cicek et al. (2004) did not find any association between the GGC repeat length and prostate cancer risk.

The PSA gene is a target of the AR. For PSA gene transcription to occur, the AR must

interact with ARE I in the PSA gene promoter. It has been mentioned that the PSA gene homozygote for the G allele is associated with a higher PSA concentration and prostate cancer (Gsur et al., 2002). Wang et al. (2003) stated that the PSA polymorphism may not be associated with the risk of prostate cancer development, progression and BPH in Japanese men.

Gsur et al. (2002) reported that they found a significant influence of the ARE I PSA polymorphism on prostate cancer risk when calculating the combination of the AG and GG genotypes versus the AA genotype, suggesting that the G allele has a protective effect. When men carried one G allele, the prostate cancer risk decreased about 63%. Otherwise, in a case analysis according to the Gleason score, the GG genotype was associated with a more aggressive disease phenotype. PSA seems to have stimulatory effects on prostatic cell proliferation.

Xue et al. (2000) reported in a study of non-Hispanic white men, comprising 57 prostate cancer cases and 156 controls, that subjects with the GG genotype were at significantly increased risk for advanced but not localized prostate cancer. After cross-classification by PSA and AR genotypes they found that men having the PSA GG genotype in combination with short CAG alleles, defined as ≤20 CAG repeats, have a ≥5-fold increase of prostate cancer risk. The frequency of the AA polymorphism is low in the normal population (5%) (Wang et al., 2003; Liu et al., 2003); however, it was found to be high in individuals diagnosed with prostate cancer (43%) (Giovannucci et al., 1999; Medeiros et al., 2002). We did not observe a statistically significant difference between controls, BPH and adenocarcinoma for the PSA A/G polymorphism ($P = 0.483$; $P > 0.05$). Furthermore, we also did not find a statistically significant difference between the PSA A/G polymorphism and CAG trinucleotide repeats in the AR gene. However, we observed higher GG variant frequencies in the short CAG repeats compared to AA and AG variants. These data were in accordance with the findings of Xue et al. (2000).

In conclusion, results of our studies conducted on prostate cancer patients in Cukurova region may imply that the PSA A/G polymorphism is not related to prostate cancer; however, CAG trinucleotide repeat expansion size is adversely related to prostate cancer risk.

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