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RESEARCH ARTICLE

Androgen Receptor CAG repeat Length polymorphism Is Not Associated with the risk of Benign Prostatic Hyperplasia

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Abstract

Benign prostatic hyperplasia (BPH) is a very frequent age-related proliferative abnormality in men. The androgens, particularly DHT regulate the growth of prostatic epithelial cells by binding to the androgen receptor (AR) present in nucleus. The DHT-AR complex interacts with specific target promoter sequence regulating transcription of target genes. Polymorphic CAG repeat in the androgen receptor (AR) can alter transactivation of androgen-responsive genes and potentially influence BPH risk. We analyzed the association between CAG repeat length and risk of BPH in a case-control study of a Indian population. We evaluated 217 subjects; 128 with BPH and 89 healthy controls. DNA was extracted from peripheral leucocytes and the AR gene was analyzed by DNA sequencing followed by PCR. We have analyzed common polymorphisms in this gene in 128 BPH patients, and 89 controls. We didn't observe the mean CAG repeats length has significant difference between cases and controls. We also categorized the data in three different form (Short<17, Moderate17-19 and Long >19), but the frequency distribution of CAG repeat was no significant between cases and controls. Also no significant difference was observed with extreme and moderate size (Extreme<17 &>19 and Moderate 17-19) between cases and controls.

Introduction

Benign prostatic hyperplasia (BPH) is a very frequent age-related proliferative abnormality in men (Kirby RS., 2000). BPH is considered a progressive disease, defined as continuous growth of the prostate, leading to intensification of symptoms and increased risk of complications, such as increased risk of acute urinary retention and BPH-related surgery (Carson C., 2003). It is one of the most common medical condition in middle aged and older males, affecting 40-50% of men by age 50 and up to 80% of men by age 70 (Kirby RS., 2000 ; Platz EA et al., 2002). Androgenic stimulation is one of the most important factor that contributes to the pathogenesis of this condition. Specifically, the development of BPH epithelial nodules requires the presence of androgens (Price H et at., 1990). As prostatic nodules enlarge, mechanical obstruction of urinary outflow may occur, leading to symptoms of urinary hesitancy, frequent urination, disuria (painful urination), and increased risk of urinary tract infection. The androgens, particularly DHT regulate the growth of prostatic epithelial cells by binding to the androgen receptor (AR) present in nucleus. The DHT-AR complex interacts with specific target promoter sequence regulating transcription of target genes. The AR gene is located at Xq11.2-q12 and is more than 90 kb in length (Feldman D., 1997; Cussenot O et al., 1998). The open reading frame is separated over eight exons. Exon 1 of the AR gene,

situated in the X-chromosome, contains a polymorphic CAG repeat sequence that encodes a variable length polyglutamine chain in the transcriptional activation domain of the AR. AR gene is the most important risk factor for prostate cancer (CaP) and benign prostate hyperplasia (BPH) in virtually all populations studied. In vitro studies have shown a negative correlation between the number of CAG repeats and the transcriptional activity of the AR. The increased number of these repeats reduces transcriptional activity in the AR, whereas a reduction to zero induces increased AR (Chamberlain NL et al., 1994; Beilin J et al., 2000; Ding D et al., 2005). It is believed that the Shorter AR gene CAG repeat lengths also have been shown to be related to a higher risk of BPH and prostate cancer development or progression (Coetzee GA et al., 1994; Giovannucci E et al., 1997; Stanford JL et al., 1997; Ingles SA et al., 1997). Short CAG repeat lengths have also been associated with androgenetic alopecia (Sawaya ME et al., 1998; Ellis JA et al., 2001), ankylosing spodylitis (Mori K et al., 2000), mental retardation (Kooy RF et al., 1999), younger age of male rheumatoid arthritis (Kawasaki T et al., 1999), and hepatistis B related hepatocellular carcinoma (Yu MW et al., 2000). In contrast extremely long AR CAG length (i.e; 38-52 repeats) has also been associated with Kennedy's disease, spinal and bulbar muscular atrophy (SBMA) (La Spada AR et al., 1991; Kuhlenbaumer G et al., 2001).

Therefore, the purpose of this present study was to investigate whether CAG repeat length polymorphism can be related to the development of BPH analyzing the frequency of AR CAG polymorphism in a sample of male individuals from India.

Materials & Methods:

Case-control study:

Subjects: We recruited 217 subjects which included 128 BPH (age range: 45–80 years, mean 65.5 years), and 89 control subjects (age range: 45– 75 years, mean 63.5 years) from the Department of Urology, King George's Medical University, Lucknow during the period of 2008–2012. Inclusions criteria of BPH were age >45 years, patients with LUTS and weight >25 g prostate size on ultrasonography (USG), American Urological Association symptom score >7, prostate specific antigen (PSA) <4.0 ng/ml, and an enlarged smooth prostate on digital rectal examination (DRE), those between 4.0 and 10 ng/ml PSA were included only if 10 core trans rectal ultrasound (TRUS) biopsy were negative. Controls were healthy volunteers (staff employee of the institute and attendants of other patients visiting the hospital for minor non-prostatic medical and surgical problems, after their informed consent), >45 years of age with normal DRE, PSA <4 ng/ml, and with no LUTS and negative family history of PC. The subjects were recruited after informed written consent in response to a fully written and verbal explanation of the nature of the study. The study was approved by the Institutional Ethical Committee (IEC), of the King George's Medical University, Lucknow.

DNA isolation

Genomic DNA was isolated from the peripheral blood lymphocytes of subjects using the Phenol-Chloroform-Isoamyl method. Briefly, four volumes of buffer A (1M Tris HCl + 1M Sucrose + 100mM $MgCl_2$ + 1% Triton-X) were added to the blood sample in 15 ml falcon tube, mixed well for five minutes, followed by centrifugation at 8000 rpm for 15 minutes. Supernatant was carefully discarded by tilting the tube, followed by addition of 0.4 volumes of buffer B (1M Tris HCl + 0.5M EDTA + 5M NaCl), 0.1 volume of buffer C (5M Sodium acetate), 0.1 volume of 10% SDS, and 1.2 volume of PCI [Phenol (25):Chloroform(24):Isoamyl alcohol (01)]. The mixture was incubated at 55 ^{0}C for two hours in a water bath without shaking. The suspension was centrifuged at 12000 rpm for 15 minutes to take out the upper aqueous layer of the supernatant. To this, 0.6 volumes of chloroform were added followed by centrifugation at 12000 rpm for 15 minutes. The upper aqueous layer was separated without disturbing the chloroform layer. To this, two volumes of chilled isopropanol were added, followed by gentle mixing until the DNA threads were visible. The contents were then centrifuged at 12000 rpm for 15 minutes to settle down the precipitated DNA. The DNA pellet was washed two times with 70% ethanol by centrifugation at 8000 rpm for eight minutes. The DNA pellet was left to air dry, followed by addition of 200 µl 1X TAE buffer to dissolve the DNA. After quantitation, DNA samples were diluted to working concentration (10ng/µl) in water. DNA concentration was determined using spectrophotometric method by reading absorbance at 260 nm, followed by dilution to 10ng/µl (working concentration) in standard TE buffer.

PCR Assay for CAG Repeat Analysis

CAG repeat motif of exon 1 in the AR gene was amplified using a pair of primers flanking

the CAG repeat motif (Cram et al., 2000). Primers were synthesized in an ABI 392 Oligo synthesizer (Perkin Elmer, Foster City, Calif). The forward primer was synthesized with 59 FAM (carboxy-fluorescein) label (Perkin Elmer) in order to analyze the PCR product in the automated DNA sequencer (ABI 377). The forward primer was also synthesized without a fluorescent label for sequencing. Polymerase chain reaction (PCR) of each sample was performed in a 0.2 mL thin wall tube using 5.0 ng of DNA, 10 pM of each primer, 200 mM deoxynucleotide triphosphates, 13 PCR buffer containing 1.5 mM $MgCl_2$, and 2 units of AmpliTaq Gold (Perkin Elmer). Amplification was carried out in a Gene Amp 9600 thermal cycler (Perkin Elmer) at 94 ^{0}C for 10 minutes, and then 30 cycles at 94 ^{0}C for 1 minute, 60 ^{0}C for 1 minute, 72 ^{0}C for 1 minute, and a final extension at 72 ^{0}C for 5 minutes.

GeneScan and Genotyping

Samples (PCR products) were prepared by mixing 1.0 mL of PCR products, 1.5 mL of loading dye (formamide:blue dextran; 5:1) and 0.5 mL of GS-ROX500 (0.5 mL/sample). After denaturation (94 ^{0}C for 2 minutes), samples were electrophoresed in 5% Long Ranger (FMC) gel using an ABI 377 automated DNA sequencer (Perkin Elmer). Raw data were analyzed using GeneScan and Genotyping software programs (Perkin Elmer) to obtain the allele (repeat) size (Thangaraj et al., 1999).

Automated DNA Sequencing

To confirm the CAG repeat numbers (allele), 2 samples (PCR products obtained using nonfluorescent primers) from each repeat size were sequenced after treating them with exonuclease I and shrimp alkaline phosphatase (Amersham) at 37 ^{0}C and 80 ^{0}C (15 minutes each). Sequencing of PCR products was carried out using 50 ng (2 mL) of PCR product and 4 pM (1 mL) of nonfluorescent primer (forward and reverse separately), 4 mL of BigDye Terminator ready reaction kit (Perkin Elmer), and 3 mL of double-distilled water to adjust the volume to 10 mL. Cycle sequencing was carried out in a GeneAmp 9600 thermal cycler (Perkin Elmer) for 30 cycles at 96 ^{0}C for 10 seconds, 50 ^{0}C for 5 seconds, and 60 ^{0}C for 4 minutes. Extended products were purified by alcohol precipitation followed by washing with 70 % alcohol (Thangaraj et al., 1999). Purified samples were dissolved in 10 mL of 50% Hi-Di formamide and analyzed in an ABI 3700 automated DNA Analyzer (Perkin Elmer).

Statistical analyses

All the comparisons were done using statistical software package "STATISTICA" and the results were confirmed by online available statistical tools (www.vassarstats.net). CAG repeats length of all controls and all cases were compared by student's independent 't' test. The frequency distribution between cases and controls was compared using chi square test. Distribution of cases and controls in three allele size categories (short, average, and long allele length: <17, 17-19, >19 respectively) and in two allele size categories (Extreme and moderate size: <17 and >19, 17-19 respectively) was also compared. CAG means between groups and lengths were compared by two factors ANOVA, and the significance of mean difference within and between the groups was done by Bonferroni post hoc test after adjusting the significance for multiple contrasts. Two-sided P values of less than 0.05 (95% level of confidence) were considered significant for inference. The power of all statistical tests was 80.0 % with 5.0% margin of error.

Results

CAG alleles in androgen receptor gene were normally distributed between controls and cases (Figure 1). CAG repeat lengths were between 10 and 24 in BPH patients and between 11 and 31 in controls. Mean CAG repeat lengths between controls (mean=18.1, SD= 2.79) and cases (mean =17.5, SD=2.72) had no significant difference (t=1.36, P=0.17).

CAG repeat lengths between cases and controls were compared in three CAG allele size categories (short, moderate and long) and in two CAG allele size categories (extreme and moderate) after taking CAG repeat length into account as categorical and continuous variable.

Short, moderate and long CAG allele size (<17,17-19 and>19)

Frequency distribution of CAG repeat length between cases and controls in three CAG length categories had no significant difference ($\chi 2$=2.2, P=0.33) (Table=1). Mean distribution of CAG repeat length between cases and controls also had no significant difference (F=0.0, P=1) (Figure=2).

Extreme and moderate allele size (<17 &>19 and 17-19)

Frequency distribution of CAG repeat length between cases and controls in two CAG length categories had no significant difference ($\chi 2$=0.30, P=0.86) (Table=2). Mean distribution of CAG repeat length between cases and controls also had no significant difference (F=1.4, P=0.24) (Figure=3).

Table 1: Frequency distribution (%) of CAG repeats length in three CAG length categories (short, moderate, and long).

Groups	**N**	**Short CAG (<17)**	**Moderate CAG (17-19)**	**Long CAG (>19)**	**χ2 value**	**P value**
Controls	89	24 (26.96%)	40 (44.94%)	25 (28.08 %)	2.20	0.333
Cases	128	45 (35.15%)	56 (43.75%)	27 (21.09%)		

Table 2: Frequency distribution (%) of CAG allelic mean length in two CAG length categories (extreme and moderate)

Groups	**N**	**Extreme (<17&>19)**	**Moderate (17-19)**	**χ2 value**	**P value**
Controls	89	49 (55.05%)	40 (44.94%)	0.303	0.862
Cases	128	72 (56.25%)	56 (43.75%)		

Figure 1: Frequency distribution of CAG alleles in the BPH and Control samples

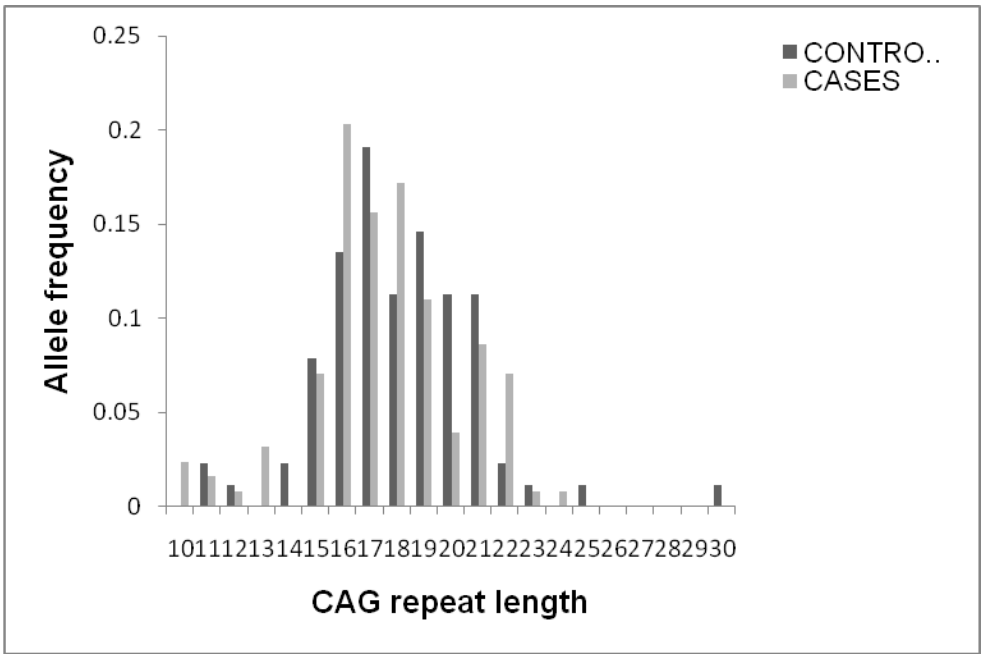
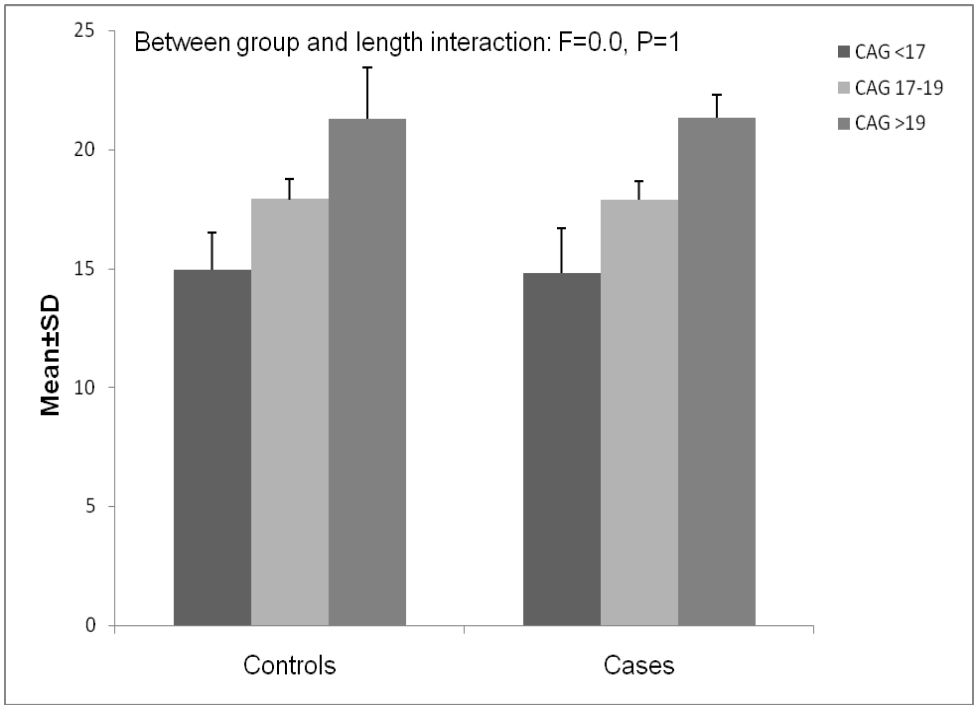
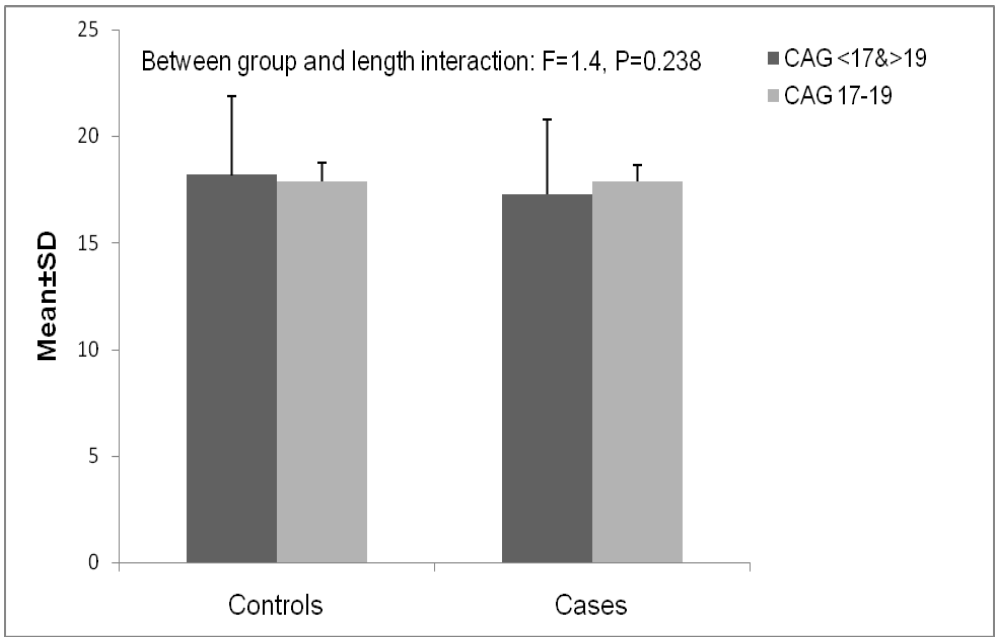


Figure 2: Comparison of allelic mean data in three CAG categories (Short, moderate and long alleles) between controls and cases



*Two factorial ANOVA was used for comparison between controls and cases

Figure 3: Comparison of mean CAG repeat length in two CAG length categories (extreme and moderate alleles) between Controls and Cases.



*****Two factorial ANOVA was used for comparison between controls and cases

Discussion:

Our results showed no association between AR CAG allele length and risk of developing BPH. The AR is a transactivation factor that depends on the binding of a steroid hormone. This androgen-regulated transactivation activity is a key factor in the proliferation and differentiation of prostate cells. The polymorphic variation of the AR gene regulatory region, where the polymorphisms with the highest variation are located (CAG and GGC), may alter the transcriptional activity of the receptor (Chamberlain NL et al., 1994). On the basis of these assumptions, Coetzee and Ross (Coetzee GA., Ross RK ., 1994) hypothesized that variation in transcriptional activity of AR, related to polymorphic CAG repeats, influences BPH and prostate carcinogenesis.

In the present study, we found no significant difference in the number of CAG repeats between the control and BPH groups. The few studies that investigated AR CAG repeat and the risk to develop BPH have shown conflicting results. Our findings support some data from the literature that also did not demonstrate differences between CAG repeat means in BPH patients and controls (Bousema JT et al., 2000; Mononen N et al., 2002; Schatzl G et al., 2002). AR CAG repeats were not associated with the risk to develop BPH, but shorter AR CAG repeats and PSA non-GG genotypes were significantly associated with decreased risk in BPH patients (Das K et al., 2008). Three large studies have examined AR CAG repeat length and risk of clinically significant BPH; two reported a significant inverse association (Giovannucci EL et al., 1999; Giovannucci E et al., 1999) and the other a significant positive association (Mononen N et al., 2002). However, in a recent case-control study of 416 BPH cases and 527 controls, CAG repeat length was associated with the risk of incidence of BPH (Kristal AR et al., 2008).

It is believed that shorter AR CAG repeat impose a higher transactivation activity on the receptor and have an increased binding affinity for androgens (Feldman D., 1997; Coetzee GA et al., 1994). This may make the prostate more vulnerable to chronic androgen over stimulation and increased proliferative activity, which in turn could increase the rate of somatic mutation among tumour supressor gene (eg. AR). This was supported by a case control study conducted by Mishra et al in 2005 (Mishra et al., 2005), which reported a 1.12 fold increase in prostate cancer risk with each shortening of a CAG repeats. Mean CAG repeat was significantly lower in CaP patients as compared to the controls and BPH patients. They repoted that individuals with ≤ 22 repeats have 2.9 times greater risk for CaP than those with >22 CAG repeats. They also analysed the CaP risk by grouping the CAG repeats in tertiles according to controls and found an increased risk of CaP with short and intermediate groups as compared to longer CAG repeat tertile.

Another study (Mitsumori K et al., 1999) in Japanese population reported association of shorter CAG repeat length with large BPH size. Contrary to this, no association was found between CAG repeat polymorphism and risk for BPH in Dutch population (Balic I et al., 2002).Unexpectedly, short CAG repeats were significantly less common in BPH patients in Finnish population (Mononen N et al., 2002).

Several case-control studies have investigated the relationship between CAG repeat length in the AR and BPH with contradictory findings. In our results, the mean CAG repeats length has no significant difference between cases and controls. We also categorized the data in three different form (Short<17, Moderate17-19 and Long >19), but the frequency distribution of CAG repeat was not significant between cases and controls. Also no significant difference was observed with extreme and moderate size (Extreme<17 &>19 and Moderate 17-19) between cases and controls. Our results are in accordance with some of these studies in which no overall significant association between CAG repeat length and BPH risk was found, whereas other studies found at least a moderate increase in risk with short CAG repeats.
After considering all the studies cited in this article we conclude that a relatively very large sample size of the subjects are required to predict involvement of CAG repeat length in BPH and this could be an important step towards understanding exact mechanism of AR CAG polymorphism in BPH risk.

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Conflict of interest

None of the contributing authors have any conflict of interest, including specific financial interests or relationships and affiliations relevant to the subject matter or materials discussed in the manuscript.

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