

Length and somatic mosaicism of CAG and GGN repeats in the androgen receptor gene and the risk of prostate cancer in men with benign prostatic hyperplasia

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Background: The most common malignancy in men worldwide is cancer of the prostate and determinants of prostate cancer (PRCa) risk remain largely unidentified. Many candidate genes may be involved in PRCa, such as those that are central to cellular growth and differentiation in the prostate gland. We analysed the polymorphic CAG and GGN repeats sequence in exon 1 of the AR gene to determine if the number of repeats might be an indicator of PRCa risk in patients with BPH.

Methods: The study evaluated 28 patients who presented with PRCa at least 6 years after the diagnosis of BPH and 56 matched patients with BPH who did not progress to PRCa over a comparable period.

Results: This study showed no evidence for association between the size of AR CAG and GGN repeats and the risk of the development of PRCa in patients with BPH. However, BPH patients with AR CAG instability had a 12-fold increased risk in development of PRCa.

Conclusions: While independent confirmation is required in further studies, these results provide a potential tool to assist prediction strategies for this important disease.

Key words: Androgen receptor, prostate cancer, polymorphism, benign prostate hyperplasia, instability, triplet repeat instability

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Accepted for publication: September 2003

Ann Saudi Med 2004; 24(1): 21-26

Prostate cancer (PRCa) constitutes a major health issue worldwide, and is now considered one of the most common causes of cancer death in men.^{1,2} The etiology of PRCa is unclear, although current evidence suggests that PRCa is likely caused by complex interactions among genetic, endocrine and environmental factors.³⁻⁵ Benign prostatic hyperplasia (BPH) is a non-neoplastic enlargement of the prostate. BPH is extremely common, with a rapid increase in prevalence in the fourth decade of life. According to epidemiological studies, most cancers arise in prostates with BPH (83.3%).^{6,7} Compared to those without BPH, men with the condition have a five-fold greater risk of developing PRCa and a four-fold greater risk of death from PRCa.⁸ A previous study reported that a family history of prostate disease (PRCa or BPH) was more frequently seen in relatives of men with BPH (20%) than in relatives of men with PRCa (12.8%) or in healthy controls (5.1%).⁹ In addition, *in vitro* malignant transformation of BPH tissue has been previously reported.¹⁰⁻¹² With the increasing incidence of BPH in the ageing population, there is an urgent need for the identification of molecular markers that can serve as prognostic indicators for developing PRCa in those patients with BPH.

Androgens regulate growth and differentiation of the prostate cells through binding to a nuclear androgen receptor (AR). The AR gene is located on the long arm of the X chromosome (Xq12-13).^{13,14} The first exon of the AR gene contains two polymorphic trinucleotide repeat segments, a CAG and a GGN, which encode polyglutamine and polyglycine tracts, respectively. The number of CAG and GGN repeats varies in the general population. Longer CAG repeat lengths appear to result in reduced AR transactivational activity both *in vivo* and *in vitro*.^{15,16} Chamberlain and coworkers¹⁵ demonstrated that the elimination of the CAG repeats in both human and rat AR resulted in a marked elevation of transcriptional activation activity. It is possible, therefore, that shorter CAG alleles result in more rapid growth of prostate cells, which in turn elevates the risk of PRCa. The functional consequences of variation in the GGN tract are less clear.

Shorter AR CAG repeats, and thus a more transcriptionally active AR, have been associated with increased PRCa risk,⁷⁻²² progression,²³ earlier age of cancer onset in white men,^{24,25} and aggressive early stage PRCa.¹⁸ Other investigators found no such association between short CAG alleles and PRCa.²⁶⁻²⁹ Shorter AR GGN repeats have also been associated with an increased PRCa risk,^{18,20,27} but other studies found no such association.^{22,26,28} In

addition, epidemiological studies have shown that a shorter length of both CAG and GGN repeats confers a higher risk of PRCa.^{18,20}

Investigators found an inverse correlation between CAG repeat length and prevalence of symptomatic BPH.³⁰⁻³² Giovannucci *et al.*³¹ found that men with repeat lengths of 19 or less had a 76% higher incidence of BPH surgery than men with repeat lengths of 25 or more. However, another study found no association between the CAG repeat polymorphism in the *AR* gene and the risk of BPH.^{33,34}

Somatic mosaicism, triplet repeat instability (TRI) of the *AR* CAG repeat in somatic cells within individual PRCa patients has been previously reported.^{35,36} Whether instability of the CAG repeat itself conferred a growth advantage on the cells in which it occurred is not known.

We analyzed the *AR*, CAG and GGN repeats to determine if the length of repeats is associated with a risk of developing PRCa in patients with BPH and if TRI of these two markers is implicated in the risk of PRCa development in BPH patients.

Methods

This study took advantage of a comprehensive population-based health care system to identify a well-defined case-control study nested within a cohort with BPH (1974-1990). Details of the study have been described previously.³⁷ Briefly, paraffin-embedded tissue samples were obtained from 28 Scottish men with biopsy proven BPH who were subsequently diagnosed with biopsy proven PRCa at least 6 years (range, 6-15 years) after the initial BPH sample. The control group consisted of 56 men who had biopsy proven BPH (with no malignant histology) on 2 separate occasions, at least 6 years apart (range, 6-15 years). Two biopsies for each patient were examined: BPH and PRCa samples in the case population and two BPH samples in the control population. All sections were re-reviewed by two pathologists to confirm the diagnosis.

DNA extraction

DNA was extracted from the BPH sample of the case group and both BPH samples of control patients. For malignant samples, laser capture microdissection (LCM) was used to obtain tumor for DNA extraction.^{38,39} Normal epithelial cells were also microdissected for samples showing somatic mosaicism.

PCR amplification

PCR primers were designed to amplify a 300bp fragment around the *AR* CAG repeat (forward primer 5'-TCC AGA ATC TGT TCC AGA GCG TGC-3' and reverse primer 5'-FAM-GCT GTG AAG GTT GCT GTT CCT CAT-3') and a 180bp fragment around the *AR* GGN repeat (forward primer 5'-ACA GCC GAA GGC CAG TTG TAT-3' and reverse primer 5'-TET-AGG AAA GCG ACT TCA CCG CAC CT-3'). A GC-rich PCR system kit (Roche, Lewes, UK) was used to improve amplification of the GC-rich portion of AR exon 1. DNA (100-500ng) was

subjected to PCR amplification in a 50µL reaction mixture containing 200µM dNTP mix (Bioline, London, UK), 10pmol of each primer, 5M of GC-rich resolution solution, 5× GC-rich PCR reaction buffer, 2 unit GC-rich enzyme mix and PCR grade water. DNA was initially denatured at 94°C for 2 minutes and thereafter subjected to 35 cycles of PCR amplification with denaturation for 1 minute at 94°C, annealing for 2 minutes at 60°C, extension for 2 minutes 30 seconds at 72°C, and final extension at 72°C for 10 minutes. For assessment of repeat instability status, BAT26 marker and the Huntington CAG (HD) repeat region were amplified in those samples showing TRI for the *AR* CAG repeat. BAT26 marker contains a polyA repeat region within exon 5 of the *hMSH2* gene. This region was amplified using primers BAT26 (forward primer 5'-TGA CTA CTT TTG ACT TCA GC-3' and reverse primer 5'-FAM-AAC CAT TCA ACA TTT TTA ACC C-3'). The HD protein contains a polymorphic CAG repeat. Efficient amplification of the HD CAG repeat was achieved using primers flanking the CAG repeat region.⁴⁰ The PCR amplification for BAT26 region was carried out in a 25 µL reaction mixture containing 10× PCR buffer (MBI, Sunderland, UK), 2.5mM MgCl₂ (MBI), 200 µM dNTP mix (Bioline, London, UK), 10 pmol of each primer, 1 unit of Taq polymerase (Roche, Lewes, UK), and sterilised distilled water. DNA was subjected to 35 cycles of PCR amplification as shown above. For the HD CAG repeat region, PCR was carried out in a 25 µL reaction mixture containing 10× PCR buffer (Boehringer, UK), 1.5 mM MgCl₂ (Boehringer, UK), 200 µM dNTP mix (Bioline, London, UK), 2.5 µL DMSO (Sigma, UK), 10 pmol of each primer, 1 unit of Taq polymerase (Roche, Lewes, UK), and sterilised distilled water. DNA was then denatured at 94°C for 4 minutes and thereafter subjected to 35 cycles of PCR amplification with denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, with extension for 45 seconds at 72°C, and final extension at 72°C for 10 minutes.

Size determination of markers studied

The sizes of PCR products and repeats number for *AR* CAG, *AR* GGN, *HD* CAG, and *BAT26* were determined using an ABI PRISM 310 Genetic Analyser (Perkin Elmer, Applied Biosystems, Warrington, UK). In cases of samples showing TRI the range of expanded alleles was defined as the number of peaks giving more than 5% of the height of the main peak (the highest peak).^{41,42} In cases where two peaks observed at the highest point of the trace, the midpoint was recorded.⁴¹ The reproducibility with which the repeat size could be determined was estimated by comparison of samples between different ABI-310 runs. In each case, both the PCR amplification and the ABI-310 analysis were repeated at least twice.

Table 1. Somatic mutation for CAG repeat lengths in case and control groups.

Population	Large to small allele size (%)	Small to large allele size (%)	Combined changes (%)	Unchanged (%)
Case (n=11)	8 (31)	3 (11)	11 (42)	15 (58)
Control (n=18)	6 (11)	12 (21)	18 (32)	38 (68)

Table 2. Somatic mutation for GGN repeats length in case and control groups.

Population	Large to small allele size (%)	Small to large allele size (%)	Combined changes (%)	Unchanged (%)
Case (n=5)	4 (15)	1 (4)	5 (19)	21 (81)
Control (n=15)	12 (21)	3 (5)	15 (27)	41 (73)

Table 3. CAG repeats numbers in samples showing TRI for AR CAG marker in case and control groups.

Samples	CAG repeat numbers (S)		
	First biopsy	Second biopsy	Normal epithelial cells
Case			
1	19, 24 (22)	19, 24 (22)	22 (22)
2	19,21 (20)	—	20 (20)
3	20, 24 (22)	20, 24 (22)	22 (22)
4	22, 26 (24)	21, 22, 24, 25, 26 (24)	27 (27)
5	19,20,22,24,25,26 (22)	19,20,22,24,26 (22)	24 (24)
Control			
1	19,23 (23)	19,23 (23)	x

(S)=the CAG repeat recorded for the sample, — =samples difficult to microdissect, x=samples not obtained.

Statistical analysis

The Wilcoxon test was used to compare AR triplet repeat variation between the 1st and 2nd biopsies of patients in the case and control groups (using SPSS version 9). The Mann-Whitney test was used to determine whether there was a significant difference, according to the number of AR triplet repeats, between case and control groups (SPSS version 9). The frequency of CAG and GGN somatic changes in the case and control populations were compared statistically using the chi-square test (Minitab version 12.1, Minitab Inc., Coventry, UK). The odds ratio for risk of PRCa with AR CAG was calculated using Stata 1.0 software. The difference in proportion test (two independent samples) was used to compare the frequency of CAG TRI between case and control populations.

Results

Comparison of AR CAG repeat length in case and control populations

CAG repeat number was determined in all BPH samples (for case and control groups) and in 26 (of 28 samples) of the PRCa samples, as two malignant samples were difficult to microdissect. In these two samples the malignant foci were small and located on the edge of the haematoxylin and eosin slide, but these foci were cut off when the sections were prepared for microdissection. In the case population, the mean CAG repeat length was 22 (range, 19-31) in the first biopsy (28 BPH samples) and 22 (range, 19-26) in the second biopsy (26 PRCa samples). In the control population, the mean CAG repeat length was

21 (range, 18-29) in the first biopsy (56 BPH samples) and 22 (range, 17-29) in the second biopsy (56 BPH samples).

The CAG repeat length was not significantly different between the first and second biopsies for each patient in the case (BPH to PRCa) and control (BPH to BPH) groups (P value=0.08 and 0.72, respectively). The number of CAG repeats between the first case biopsies (BPH) and the first control biopsies (BPH) failed to reach significance at the 5% level (P value=0.25). A similar finding was observed when the number of CAG repeats were compared between the second case biopsies (PRCa) and the second control biopsies (BPH) P value=0.58.

Somatic mutation for CAG repeat length was determined in 29 patients; 11 patients from the case population and 18 from the control population (Table 1). The difference in the CAG repeat number between the first and second biopsy for the same patient ranged from 1 to 5 repeats. The frequency of CAG somatic changes from the large to small allele was significantly higher in the case group compared to the control (P value=0.025) and the risk of developing PRCa in BPH patients displaying this somatic mutation was 3.7 (OR=3.7, 95% CI=1.13 to 12.15).

Comparison of GGN repeat length in case and control populations

The GGN repeat number was determined in all BPH samples (for case and control groups) and in only 26 (of 28 samples) PRCa samples for the same reason mentioned above. In the case population, the mean GGN repeat length was 23 (range, 16-24) in the first biopsies (28 BPH samples) and 23 (range, 22-24) in the second biopsies (26 PRCa samples). In the control population, the mean GGN repeat length was 23 (range, 16-25) in the first biopsies (56 BPH samples) and 23 (range, 16-25) in the second biopsies (56 BPH samples).

The GGN repeat length was not significantly different between the first and second biopsies for each patient in the case (BPH to PRCa) and control (BPH to BPH) groups (P value=0.18 and 0.06, respectively). The number of GGN repeats were compared between the first case biopsies (BPH) and the first control biopsies (BPH) and the results failed to reach significance at the 5% level (P value=0.49). A similar finding was observed when the number of GGN repeats were compared between the second case biopsies (PRCa) and the second control biopsies (BPH) (P value=0.94).

Somatic mutation of the GGN repeat length was determined in 20 patients; 5 patients in the case population and 15 in the control population (Table 2). The difference in the GGN repeat number between the first and second biopsy for the same patient ranged from 1 to 4 repeats. Results showed that there were no significant differences between the case and control groups with respect to GGN changes from large to small allele (P value=0.52), small to large allele (P value=0.77), or in combined changes (all samples showing GGN somatic changes vs. all samples not showing CAG changes, P value=0.46).

Triplet repeat instability in the CAG and GGN repeats

Since the *AR* gene is located on the X chromosome, there is only a single allele per cell in the male; this accounts for the presence of a single major PCR product from each specimen. Among case and control samples screened by *AR* GGN marker, no instability was detected. TRI was determined for the *AR* CAG marker in the first and the second biopsies of 5 patients in the case population and one patient in the control (Table 3). DNA from microdissected normal epithelial prostate cells were obtained from case sections that showed CAG TRI (5 samples) and these samples showed no CAG instability (Table 3). A significant difference was found between case and control populations with respect to *AR* CAG TRI (5 patients in the case versus 1 in the control, P value=0.008) and the OR of developing PRCa was 11.95 in patients with BPH displaying instability in the *AR* CAG locus (95% CI=1.32 to 108.07). Samples showing instability for *AR* CAG markers were further screened with HD CAG and BAT26 markers. No instability was detected for either marker.

Discussion

Both PRCa and BPH are common diseases that increase in incidence with age. Previous studies have defined a significant association between BPH and developing PRCa.^{7,8} With the increasing incidence of BPH in the ageing population, there is an urgent need for the identification of molecular markers that can serve as prognostic indicators for developing PRCa in patients with BPH. Germ line and somatic variation in genes directly involved in regulation of prostate cell growth might be critically important in understanding the carcinogenesis of PRCa, as these variants might be used as diagnostic, preventative, and prognostic markers for PRCa. Our previous studies showed that a constitutive *CYP3A4* and *VDR TaqI* SNPs are associated with a group of men with BPH that are at an increased risk of PRCa and may be a useful component of a polygenic prediction strategy for this important disease.^{37,43,44} This study analysed the *AR*, CAG and GGN repeats to determine if the length/ or TRI of repeats are associated with a risk of developing PRCa in patients with BPH. Such an approach is likely to also lead to improvements in survival. In the converse situation, those patients who do not have a high risk of developing PRCa could be offered standard follow-up monitoring.

The mean number of CAG and GGN repeats observed in this study is consistent with previous studies where the number of CAG and GGN repeats cluster around 22 and 23 respectively.^{20,22-24,30,31} This study showed no association between shorter CAG and GGN alleles and risk of developing PRCa. There are a number of possible factors that may have contributed to the lack of an observed association between CAG and GGN alleles and a risk of developing PRCa in this study. The effect of shorter *AR* alleles on PRCa, as determined

in two case-control studies, is relatively small^{17,20} and the lack of a uniform model of analysis also makes these studies difficult to compare. Moreover, the control group used in this study were BPH patients, and *AR* polymorphic repeats were previously reported as associated with the development of BPH. Determining the repeat length of CAG and GGN in age-matched healthy controls would be useful to establish whether the *AR* polymorphic repeats were associated with development of BPH and PRCa in these groups or whether *AR* polymorphic repeat length is of no use as a prognostic marker for the development PRCa in patients with BPH.

In this study, somatic mutation with respect to the size of *AR*, CAG and GGN, markers were investigated in two different biopsies from the same patient. The results suggest that patients with CAG repeat lengths changing from a large (in the first biopsy) to a small allele (in the second biopsy) are at four times greater risk of developing PRCa than those where this does not occur (Table 1). This mutation might be involved in the process of developing PRCa. This study also showed a significant difference between the case and control population with respect to *AR* CAG TRI. The OR of developing PRCa in BPH patients having TRI in the *AR* CAG repeats was 11.95 (95% CI=1.32 to 108.07). PCR amplification of this region of the gene showed only one CAG allele from normal prostate epithelial cells. The high relative incidence of the CAG instability in benign and tumour cells suggest that the

TRI occurred in cells with a growth advantage. If the CAG instability had occurred in cells without a growth advantage, it would not have been detected, as its presence would be obscured by the proliferation of cells with a growth advantage. However, whether the CAG instability conferred a growth advantage on the cells in which it occurred is not known. Amplification of the independent polymorphic markers, *HD CAG* and *BAT26*, from benign tumour DNA was used in this study as a negative control as both markers play no known or suspected role in prostate neoplasia.

This study showed no evidence for an association between the size of *AR* CAG and GGN repeats and the risk of the development of PRCa in patients with BPH. However, the results suggest that CAG somatic changes from the large to small allele might have a role in development of PRCa. The study shows that a constitutive *AR* CAG instability is associated with a group of men with BPH that are at an increased risk of PRCa. This instability may be a useful component of a polygenic prediction strategy for this important disease. Although there was no obvious bias in the sample set used, the present results and conclusions might be regarded as tentative due to the relatively small sample size.

Acknowledgements

This work was supported in part by the UK Saudi Cultural Office.

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