Original Article

Androgen Receptor: CAG Repeat Length and Transcriptional Levels Related to Prostate Cancer

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Abstract

Objectives: The present work evaluates variations of polymorphic [CAG]n repeats present at exon 1 of the AR gene, as well as relative levels of its transcript, in order to investigate associations of these factors with prostatic tumor genesis in the Brazilian male population. **Methods**: Genomic DNA was extracted from blood samples from patients with prostate cancer (PCa), benign prostatic hyperplasia (BPH), and from a group of young Brazilian males to determine the number of [CAG]n repeats amplified by PCR. Mutation analysis in this amplified fragment was carried out using the LIS-SSCP technique. Total RNA was extracted from prostatic tissue to evaluate the AR gene transcript levels using semi-quantitative multiplex RT-PCR. **Results**: CAG length varied from 14 to 30, with an average of 21 repeats for PCa and the male group and 20 for the BPH group. No significant difference was found for [CAG]n polymorphism among the analyzed groups and there was no sporadic change in the amplified portion of the AR gene, nor loss of [CAG]n repeats, demonstrating that these do not contribute to the cancer occurrence. Nevertheless, the positive association between short alleles and TNM pT3 staging may indicate that CAG repeats is associated to PCa progression. The transcriptional levels were significantly increased in PCa than in BPH and were associated with serum PSA levels of 5-10 ng/mL. As diagnostic clinical parameter, the levels of AR gene presented 17-fold higher chance for PCa occurrence, 60% of sensibility and 95% of specificity. **Conclusion**: The data suggest that the highly miscegenated Brazilian male population presents a high frequency of [CAG]n short repeats, which may be associated with the PCa progression, while AR mRNA levels seems to be a good indicator of the incidence of this pathology, being useful in clinical practice for distinguishing patients with PCa from those with BPH.

Keywords: Receptors, Androgen; Gene Expression; Prostatic Neoplasms; Prostatic Hyperplasia

Introduction

The prostate is hormone dependent and testosterone has been suggested as one of the promoters of cancer in this gland.[1,2] Androgen–receptor protein codified by the AR gene mediates the action of testosterone on the cell nucleus. In the cytoplasm of prostatic cells, testosterone is converted into dihydrotestosterone (DHT) by the enzyme 5 α–redutase.[3-5] Androgen receptors are members of the superfamily of nuclear receptors and present a polyglutamine tail in their transactivation N–terminal portion of androgen–regulated genes. This sequence of glutamine is encoded by [CAG]n repeats present in the exon 1 of the AR gene.[6-8]

A great deal of studies involving AR CAG repeat have been divergent as to the association of this polymorphism with prostate cancer (PCa). Nevertheless, data have been pointing to a higher frequency of less repeats in individuals who developed PCa in younger ages, and the size of these repeats also modifies the risk and progression of prostate cancer.[9] Variations in [CAG]n repeats in the exon 1 of the AR

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gene may have a role in the cause and the differential response to treatment of prostate adenocarcinoma and the gene activation function of androgen receptor is inversely correlated to the length of polyglutamine.[2,10-14] Several epidemiological studies have documented CAG polymorphism in different populations, [10,12,15,16] but there are few studies in the Brazilian male population.[17,18]

Besides [CAG]n repeats present in the exon 1, about 300 polymorphic changes in the AR gene can be related to the development of several pathologies such as Androgen Insensitivity Syndrome (AIS), mental retardation (MR) and breast, colorectal and prostate cancers.[2,19] Mutations occurring on exons 2 to 8 have been associated to the occurrence and progression of prostatic tumor cells growth. Inside exon 1, expansions of trinucleotides as CAG repeats and [GGN]n that encode a polyglycine tail, in the amino- terminal domain of the androgen receptor have been associated with PCa.[20,21] Though it has been observed that the short alleles in the AR CAG repeat length may be related to prostate cancer. Some authors have investigated the levels of the AR gene,[22-24] and all have demonstrated that the transcript levels of this gene are scarce and not easily detected by traditional methodologies such as Northern blotting.[22] The literature has reported the importance of AR gene expression in the maintenance of prostatic homeostasis.

It is not known exactly how androgen is involved in prostate cancer etiology. If the androgen receptor had oncogenic potential, the androgen may play a role in cancer occurrence and alternatively may be involved in prostate cancer promotion or progression by enhancing androgen-regulated processes. To investigate molecular events associated to prostatic tumor genesis in the highly miscegenated Brazilian male population, in this present work we evaluated AR CAG polymorphism as well as its transcription levels and mutation screening.

Material and Methods

Patients and Setting

This work was developed in the Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry of the Federal University of Uberlandia (UFU), from December 2004 to July 2006, and was approved by the Committee of Ethics in Research (Authorization n° 005/2001). The blood and prostatic specimens were obtained after Informed Consent by patients and collected by the medical and urological division of the Hospital of Clinics of UFU, MG, Brazil. Eighty-three samples collected from peripheral blood defined three groups of studies: 13 patients with benign prostatic hypertrophy (BPH), 22 with PCa and 48 young male individuals (<30 years) with no history of prostatic alterations. The average age for the PCa and BPH groups was 68 (±6 standard deviation, range from 57 to 76 years) and 72 years (±10 standard deviation, range from 49 to 87 years), respectively. Prostatic tissues from PCa and BPH patients were obtained after radical prostatectomy and transurethral resection (TURP), respectively. All cases were free of preoperative chemotherapy, radiation or hormonal blockage.

Extraction of Genomic DNA and RNA

Genomic DNA of patients was extracted from blood and prostatic tissue (stocked at –80°C) according to previously described proceedings.[25] Total RNA was extracted from prostatic tissue, in duplicate for the same patients, using Trizol Reagent (Invitrogen), following recommendations of the manufacturer. DNA and RNA quality was analyzed in agarose electrophoresis gel and its concentration was determined by spectrophotometry at 260nm and 280nm.

CAG Repeat Polymorphism and Mutation Screening

AR CAG repeat was amplified in a reaction containing: 1 U of Taq DNA Polymerase (Invitrogen); 1X Taq DNA Polymerase Buffer; 200μM of each; dNTP; 3.5pmoles of each forward and reverse primers;[26] 1.5 mm MgCl2, 200ng of DNA. The final volume was completed to 20μl with distilled water. The reaction was carried out in the following conditions: an initial cycle at 95°C for five minutes; ten cycles at 94°C for one minute; 63°C for one minute; 72°C for one minute; 25 cycles at 94°C for 40 seconds; 63°C for 40 seconds; 72°C for 50 seconds, and a final extension cycle at 72°C for 10 minutes. Ten microliters of the amplified product was submitted to electrophoresis in polyacrylamide gel 19:1 for estimating the number of repeats of each sample. For LIS-SSCP, the technique used for the proceedings was described elsewhere,[27] with minor modifications. Two microliters of the amplified product was denatured at 95°C for 10 minute in a solution containing 18μl of LIS buffer (10% of sucrose, 0.01% of bromophenol blue and 0.01% of xylene cyanol). Subsequently, ten microliters of each sample was applied in polyacrylamide gel

49:1. After a running time of 8 hours and 320 volts, the gel was stained by silver nitrate.

Haplotypes for AR CAG Repeat

After electrophoresis in polyacrylamide gel, the relative migrations of each sample were evaluated. Three representative samples of different haplotypes (with low, intermediate and high molecular weight) for [CAG]n repeats were cloned with Topo TA Cloning (Invitrogen) kit according to recommendations of the manufacturer. Three clones were subjected to an automated sequencing, using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit and an automated DNA sequencer ABI 377 (Applied Biosystems-Perkin Elmer). The sequences obtained by automated sequencing were aligned with the sequence of the AR gene available at GenBank/NCBI (Access number: 178893, www.ncbi.nlm.nih.gov by the program DNAStar (Seq Man). Haplotypes of sequenced clones were plotted in a linear regression graph together with the values of relative migration of each one of three controls to create the regression equation, allowing the determination of the individual haplotype of each patient.

Multiplex RT-PCR

One microgram of total RNA was used for reverse transcription at 37°C for 1 hour in a solution containing: 40U Murine Moloney Leukemia Virus Reverse Transcriptase (MMLV-RT); 10U RNASe inhibitor (Amersham Biosciences); 200μmol dNTPs; 126pmoles of random hexanucleotide primer. The final volume was completed to 20μl with DEPC-treated distilled water. Multiplex RT-PCR was carried out by adding 5μl of cDNA in a mixture containing: 1.5U de Taq DNA Polymerase (Invitrogen); 1X Taq DNA Polymerase Buffer; 200μM dNTPs; 2.5pmoles of B2M (beta-2-microglobulin) primers [28] and 10.5pmoles of AR gene primers. The total of 35 reaction cycles were carried out in the following conditions: 95°C for 5 minute; 10 cycles at 94°C for 1 minute; 60°C for 1 minute; 72°C for 1 minute; 25 more cycles at 94°C for 40 seconds, 60°C for 40 seconds, 72°C for 50 seconds, followed by a final extension cycle at 72°C for 5 minutes. The number of cycles necessary for the exponential phase was experimentally determined.

The primers used for cDNA amplification of human AR gene were design by the software Primer Design version 2.0 and produced a 464-pb fragment length: sequence for the forward primer located in exon 2, 5'-CATGTGGAAGCTGCAAGGTCT-3'; and sequence for the reverse primer located in the junction of the exons 4 and 5, 5'-GTGTAAGTTGCGGAAGCCAGG-3'. Transcripts of B2M gene were co-amplified as an internal control of RT-PCR multiplex semi-quantitative analysis for the target gene, presenting a 534-pb amplified cDNA fragment. The final volume of reaction was completed to 25μl with distilled water.

Semi-Quantitative Analysis

The amplicons obtained for AR gene target were analyzed and quantified according to its agarose sinal intensities using the Image MasterTM VDS Software, version 2.0 (Amersham Biosciences). Densitrometry readings (IOD) were submitted to a target/B2M ratio. The average semi-quantifications of duplicate analysis for each patient were calculated in order to estimate the AR mRNA levels.

Data Analysis

Statistical analyses were carried out by BioEstat software version 3.0.[29] The non-parametric Mann-Whitney U test was used for: [CAG]n repeat comparisons among PCa, BPH and the male population group; mean comparisons of the AR semi-quantitative expression between PCa and BPH groups. The ratios of AR/B2M levels for BPH and PCa tissues were given by box spot graphical representation . Spearman's correlation coefficient was performed with all clinical and laboratorial parameters: [CAG]n repeats polymorphism, AR mRNA semi-quantitative levels, patient's age at diagnosis, serum tPSA, TNM staging system and Gleason score. Fisher´s exact test was used to verify associations between [CAG] repeats (≤21 and >21) and AR gene expression (<0.6 and ≥0.6). A cut-off was established to calculate sensibility, specificity, positive predictive value, predictive negative value and accuracy, and odds ratio for AR mRNA levels. Statistical significance was considered when p< 0.05.

Results

AR CAG polymorphism haplotypes were 21 (±4) for PCa and the male population group, and 20 (±4) for BPH patients, ranging from 14 to 30 repeats. CAG repeat length less than or equal to 21 were observed in 30 out of 48 (62%) young male individuals, in 14 out of 22 (64%) PCa patients and in 10 out of 13 (77%) BPH patients. Abrupt reduction in the frequency was observed in individuals that presented repeats higher than 25 (Figure 1). No statistical difference was found for AR CAG repeat polymorphism among the groups analyzed (P> 0.05) and LIS-SSCP did not demonstrate differential profile between polymorphic bands from genomic DNA amplified from prostatic tissue in comparison to the DNA from peripheral blood of the same patient (Figure 2).
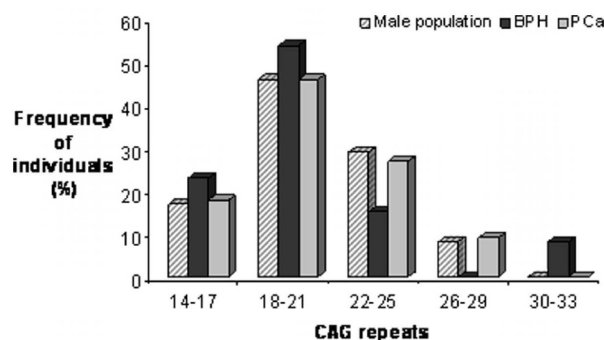
Figure 1 - Distribution of [CAG]n repeats in exon 1 of AR gene, between patients with cancer of the prostate (PCa), benign prostate hyperplasia and of a male population group
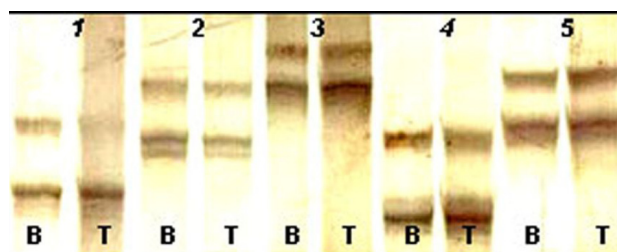
Figure 2 - LIS-SSCP profile for [CAG]n polymorphism of AR gene. From 1 to 5, we represent five different haplotypes for CAG repeats, where: "B" corresponds to the amplification of genomic DNA extracted from peripheral blood and "T" from prostatic tissue for the same patient

Correlation analysis between the number of repeats and TNM stage (r=-0.18), Gleason score (r=-0.02), pre-operative serum tPSA (r=-0.31) and patient's age at diagnosis (r=-0.06) were not significant.

The cDNA amplifications demonstrated that no prejudicial competition occurred in both profiles, separately and in a multiplex format, allowing AR semi-quantification with great efficiency and using B2M mRNA levels as endogenous control of the reaction (Figure 3).

Figure 3– Simple and multiplex RT-PCR for AR and B2M genes. 1 and 4: Amplified fragment for the target AR gene (464-pb). 2 and 3: Amplified fragment for the constitutive gene B2M (534-pb). 3 and 6: Co-amplification for genes AR and endogenous control B2M. M: 100-pb molecular weight marker

The cycling number chosen for analysis was 32 cycles due to the exponential amplification of both genes (AR and B2M).

Figures 4A and 4B represent the AR and B2M amplifications with analyses in duplicate for the same patient with BPH and PCa, respectively. RT-PCR reactions duplicated for each patient did not differ statistically for AR mRNA levels. Statistical differences were obtained for AR levels between PCa (0.61 ± 0.29) and BPH (0.37 ± 0.19) prostatic tissues with
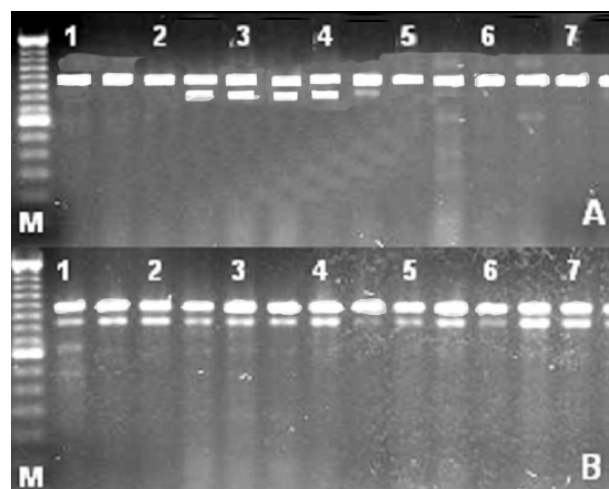
Figure 4 – Multiplex RT-PCR technique to estimate AR mRNA levels. (A) RT-PCR for AR gene from mRNA extracted from tissues of BPH and PCa patients, respectively. Both groups were represented by seven tissues samples analyzed in duplicate for each patient. M: 50-pb molecular weight marker. The B2M and AR fragments size are 534-bp and 464-pb, respectively

significantly higher in prostate cancer in comparison to benign prostatic disease (p=0.002) (Figure 5). AR expression differentiated patients with BPH from those with PCa with a cut-off equal to or higher than 0.6, where the expression of the target gene corresponds to 60% of the endogenous gene expression (Figure 5). One out 13 (8%) of BPH cases and 13 out 22 (59%) of PCa patients presented AR mRNA levels higher than
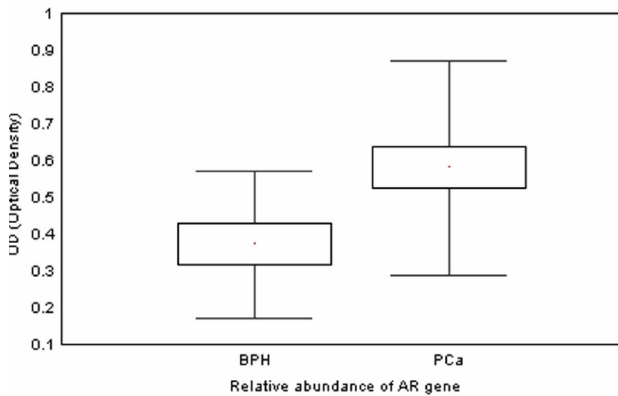
Figure 5 - Box-plot showing differences in the AR mRNA levels for benign and malignant prostatic tissues. RNA mean values and standard deviations for patients with BPH (N = 13) and PCa (N = 22) were respectively 0.37±0.19 and 0.61±0.29 (p= 0.0018)

the cut value, with 17-fold (CI 95% 1.90 − 158.0, p <0.008) chances for cancer occurrence, accuracy of 72%, sensibility of 60%, specificity of 92% and positive predictive value of 92%.

Correlation coefficients obtained between the AR gene expression and serum tPSA (r=-0.12), age of the patient at diagnosis (r=0.33), Gleason score (r =-0.03), TNM stage (r=-0.22), and [CAG]n repeats (r = 0.49) were not significant. On the other hand, positive associations were found between the AR mRNA levels ≥0.6 and serum PSA 5-10 ng/mL, and between CAG repeat length ≤ 21 and pT3 stage compared to pT2 (P= 0.04) (Table 1 and Table 2).

Additionally, a bimodal behavior for AR levels was observed among BPH group (lowest mRNA levels) and TNM stages of PCa where pT1 presented the highest AR levels (IOD = 0.81), pT2 intermediate (IOD = 0.62) and pT3 (IOD = 0.52).

Discussion

Classifications according to ethnic groups have been used in many studies to check the importance of CAG repeats in exon 1 of the AR gene associated to diseases of the prostate, including the incidence and progression of adenocarcinoma of the prostate. In these studies, the amplification of [CAG]n nucleotides has been associated to a higher risk of having PCa in black men and a lower risk in Asian men.[2,9,12,15,16,30] However, when it comes to the Brazilian population, this classification is not possible, due to a high degree of miscegenation.[17,18] The average 21 [CAG]n repeats in the exon 1 of the AR gene described here are near those of other populations, such as those described in studies carried out with North-American, [12,13] English[23] and Brazilian patients with cancer.[17] Data obtained demonstrate that these highly polymorphic repeats observed in different countries are near and regarded as

Table 1 – Distribution of [CAG]n repetition and semi-quantitative expression of gene AR between patients with BPH and clinical data

Clinical data	CAG]n repeats Number (Mean±SD; variation)		PCR SQ Number (Mean±SD; variation)	
	≤21	> 21	<0.6	≥0.6
Age	10/13 (72±7; 61-87)	3/13 (70±17; 49-81)	12/13 (71±9; 49-81)	1/13 (87)
Serum PSA				
≤4 ng/mL	3/13 (2.3±1.4; 1.1-3.8)	1/13 (3.6)	4/13 (2.6±1.2; 1.1-3.8)	ND
5-10 ng/mL	4/13 (6,0±1.9; 4.4-8.8)	ND	4/13 (6,0±1.9; 4.4-8.8)	ND
>10 ng/mL	3/13 (16.4±6.9; 11.0-24.3)	2/13 (15.2±4.6; 12.0-18.5)	4/13 (13.9±3.3; 11.0-18.5)	1/13 (24.3)

ND = no data available

Table 2 – Distribution of [CAG]n repetition and semi-quantitative expression of gene AR between patients with prostate cancer and clinical data

Clinical Data	CAG]n repetition Number (Mean±SD; variation)		PCR SQ Number (Mean±SD; variation)	
	≤21	> 21	<0.6	≥0.6
Age	8/22 (69±6; 57-75)	14/22 (67±6; 59-80)	09/22 (67±5; 61-72)	13/22 (68±6;57-75)
Serum PSA				
≤4 ng/mL	ND	ND	ND	ND
5-10 ng/mL	9/22 (7.6±1,4; 5.4-9.4)	7/22 (6.9±1,8; 5,3-9.6)	3/22 (7.2±1,2; 5.9-8.2)	11/22 (6.9±1,6; 5.3-9.6)
>10 ng/mL	5/22 (22.8 ± 14.0; 13.0-47.0	1/22 (11.1)	6/22 (22.4±12.4; 13-47)	2/22 (12.4±1,8; 13.7)
Gleason				
<7	4/22	2/22	1/22	7/22
≥7	10/22	6/22	8/22	6/22
TNM				
pT1	3/22	1/22	ND	5/22
pT2	2/22	5/22	2/22	5/22
pT3	9/22	2/22	7/22	3/22

For PCa group, elevated levels of the AR gene were associated to serum tPSA at 5-10 ng/mL compared to levels of PSA> 10 ng/mL (P=0.03), and [CAG]n repeats ≤ 21 were associated to pT3 stage compared to pT2 stage (P=0.04) (Fisher´s Exact Test). Abbreviations: SD = standard deviation; SQ = semi-quantitative; ND = no data

averages and variations to those found for the Brazilian population.[12,13,17,31,32]

The high frequency of short alleles in the analyzed population group and BPH patients did not allow the identification of possible associations of these repeats with the incidence of prostate cancer. The increased frequency of CAG short alleles in the BPH group have been observed by other studies that consider the presence of short alleles to be a genetic factor associated also to the BPH development and probably of other urological diseases.[33] Nevertheless, our results do not show a possible association of these short alleles and prostate cancer or the benign disease, and the control group was constituted by young patients, who could develop these diseases or not.

The positive association of repeats ≤ 21 with more advanced stages may indicate that short alleles to be associated to the progression of prostate adenocarcinoma, but not with its incidence, though no difference between the analyzed groups have been conclusive so as to help in the elucidation of the role of CAG repeats length in the PCa incidence in the Brazilian population.

As for the analysis of AR mutations, nearly 50% of PCa cases, including those in initial stages, contain changes in the AR gene.[2,34,35] The AR CAG portion in the exon 1 is genetically unstable and the mechanisms able to produce this instability are unknown. A possible explanation would be the incidence of slippages of DNA polymerase during the replication process.[36] The events that cause loss of heterozygosity (LOH) in the CAG polymorphism are rare and reported elsewhere as only one out of forty cases presenting deletion in CAG repeat; from 24 repeats (wild type) to 18 repeats (mutant).[37] Thus, the absence of mutations for CAG polymorphism in peripheral blood in comparison to the prostatic tissues from the same patient suggests that no sporadic mutation or no CAG triplet reduction contributed effectively to cancer development.

For studies involving gene expression, RT-PCR is quick and efficient to estimate mRNA levels from a target gene in different samples.[38,39] Reverse transcription using random hexanucleotide as primers has been very efficient for the cDNA synthesis of different genes.[38,39] The use of B2M (beta–2–microglobulin) as endogenous control presented several advantages. First, its detection after RT and PCR indicated the success of these two

steps. On the other hand, the quantity of cDNA of this gene indicates that samples are pure, the degree of degradation, and purity of mRNA extracted from the sample. Thirdly, the presence of B2M as endogenous control of RT-PCR demonstrates the variability inherent in RT process followed by PCR. Another important factor to be pointed out is that the results obtained by conventional semi-quantitative RT-PCR are generally validated by absolute quantification, meaning that the number of mRNA copies correlates significantly with comparative data. Thus, comparative analyses are appropriate and solid methods to investigate the levels of expression of a gene and they do not depend on absolute quantification.[40]

Semi-quantitative multiplex RT-PCR demonstrated a positive association between the increased levels of transcripts of the AR gene and prostate adenocarcinoma, suggesting an important role of androgen receptor in carcinogenesis. Considering the sensibility of molecular markers to cancer cell processes of transformation, patients with higher levels of the AR gene in the prostate have higher probabilities of developing adenocarcinoma of prostate.

The highest AR levels and the intermediated serum tPSA levels may be associated with the incidence or progression of prostate cancer. AR bimodal behavior in several stages of the disease suggests that the androgen receptor is one of prostate cancer promoters, due to having higher levels in pT1. Some authors suggest that the loss of AR gene expression in advanced stages may be due to the state of gene methylation, chromosomal deletion and other cellular events associated to the disease progression.[41]

Several studies have demonstrated that the *AR* gene expression remains in both, hormone-dependent and hormone-independent prostate cancers. Particularly in the hormone-independent type, *AR* levels seem to increase dramatically and this is taken to be one of the defects in hormonal blockade, probably due to gene amplification mechanisms or promiscuous activation of its protein by regulators other than testosterone, allowing tumor growth in environments with low androgen concentrations.[20,42-44]

Finally, the results of the present study suggest that the highly miscegenated Brazilian male population presents a high frequency of CAG short repeats, which may be associated to the progression of prostate cancer, but not with its incidence. Nevertheless, *AR* gene expressions seem to be a good indicator for the incidence of this pathology, being useful in clinical practice to distinguish patients with PCa from those of

BPH. All studies focused on *AR* seem to point out its role in the incidence, development and progression of prostate cancer.

Acknowledgements

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