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Rarity of DNA sequence alterations in the promoter region of the human androgen receptor gene

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Abstract

The human androgen receptor (*AR*) gene promoter lies in a GC-rich region containing two principal sites of transcription initiation and a putative Sp1 protein-binding site, without typical "TATA" and "CAAT" boxes. It has been suggested that mutations within the 5'untranslated region (5'UTR) may contribute to the development of prostate cancer by changing the rates of gene transcription and/or translation. In order to investigate this question, the aim of the present study was to search for the presence of mutations or polymorphisms at the *AR*-5'UTR in 92 prostate cancer patients, where histological diagnosis of adenocarcinoma was established in specimens obtained from transurethral resection or after prostatectomy. The *AR*-5'UTR was amplified by PCR from genomic DNA samples of the patients and of 100 healthy male blood donors, included as controls. Conformation-sensitive gel electrophoresis was used for DNA sequence alteration screening. Only one band shift was detected in one individual from the blood donor group. Sequencing revealed a new single nucleotide deletion (T) in the most conserved portion of the promoter region at position +36 downstream from the transcription initiation site I. Although the effect of this specific mutation remains unknown, its rarity reveals the high degree of sequence conservation of the human androgen promoter region. Moreover, the absence of detectable variation within the critical 5'UTR in prostate cancer patients indicates a low probability of its involvement in prostate cancer etiology.

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- Promoter region
- Prostate cancer
- 5'Untranslated region
- DNA sequence alterations

Introduction

Androgens play an essential role in prostate differentiation and in the development and growth of prostate carcinomas (1). The androgen receptor protein is essential for androgen action since it binds either testosterone or its 5α-reduced metabolite, 5α-dihydrotestosterone (2). The human androgen receptor (*AR*) gene is located in the X chromosome at position Xq11-12, comprising 8 exons and a long 5'untranslated region (5'UTR). The promoter region lacks typical "TATA" and "CAAT" boxes, but lies in a GC-rich region and contains a putative Sp1 protein-binding site (Sp1-PBS) and two principal transcription initiation sites (TIS) (3) as highly conserved elements.

The androgen receptor facilitates androgen-induced regulation of genes involved in cell proliferation and differentiation. Mutations that alter *AR* conformation, function and regulation may provide a selective growth advantage for subpopulations of cells within the tumor that are able to proliferate even in an androgen-deprived environment (4). Since the 5'UTR may play a role in translation control, protein expression may also be modulated by single-nucleotide polymorphisms (SNPs) in the *AR* promoter region, as proposed for other prostate cancer-related genes such as *CYP17* and *PSA* (5,6). So far, no SNPs have been reported in the *AR*-5'UTR (http://snp.cshl.org, 7) but Crocitto and collaborators (8) described two germ-line point mutations in the most conserved portion of the promoter region among 38 individuals with prostate cancer.

In the present study, the conformation-sensitive gel electrophoresis (CSGE) method was applied to the screening of the Sp1-PBS- and TIS-containing portion (~0.5 kb) of the androgen receptor promoter region in a sample of prostate cancer patients in order to determine the occurrence of germ-line mutations or SNPs that could possibly affect gene transcription or translation rates. The CSGE method is considered to be more sensitive than single-strand conformation polymorphism since the conformation polymorphism in the DNA heteroduplexes is enhanced by the denaturing environment, allowing the detection of variations as small as SNPs (9). Since human males are hemizygous for the *AR* gene, we adapted the CSGE method by the addition of corresponding polymerase chain reaction (PCR) products from previously sequenced control DNAs. Furthermore, CSGE was used for the analysis of a larger *AR*-5'UTR (~1.3 kb) in blood donors from a Brazilian population in order to assess its variability.

Material and Methods

Blood samples were obtained from 100 healthy male blood donors at Hemocentro, State University of Campinas, Campinas, SP, Brazil. The ages of these individuals ranged from 18 to 58 years (mean ± SD: 36.6 ± 9.7 years). Genomic DNA samples from 92 prostate cancer patients were obtained from the Urology Outpatient Clinic of the School of Medicine of São José do Rio Preto, SP, Brazil. The diagnosis of adenocarcinoma of the prostate was established by one of the authors (J.C. Mesquita) by histological examination of specimens obtained from transurethral resection of the prostate or after prostatectomy. The ages of these patients ranged from 43 to 96 years (mean ± SD: 73.6 ± 9.2 years). The same patients have been previously evaluated concerning the CAG repeats and *Stu*I polymorphism of the *AR* gene and 5'promoter region polymorphism of the *CYP17* gene (10,11).

Table 1. Primer sequences used to amplify the androgen receptor 5'UTR promoter region in blood donors and prostate cancer patients.

Primers	Sequence (5'→3')	Position (*)	Fragment size (bp)	Annealing temperature	References
1S	GTGAGTGCTGGCCTCCAGGA	–294 to –272	425	64ºC	Present paper
2AS	GAGGTCACGACATGTCCTCGGC	+109 to +131			
3S	GTTGCATTTGCTCTCCACCTCCC	–23 to –1	263	60ºC	Present paper
4AS	TCACCGAAGAGGAAAGGGCAGCTC	+216 to +240			
5'UTR2-S	CTTCGAAGCCGCCGCCCGG	+197 to +215	350	62ºC	Crocitto et al. (8)
5'UTR-AS	TTCTTCCGAGAATCCTCGGT	+526 to +546			
7S	TGCAGAGAGGTAACTCCCTTTG	+472 to +494	490	64ºC	Present paper
8AS	CGCTCTGAGAGCCTCAAAGTC	+941 to +962			

AS = antisense; S = sense; 5'UTR = 5'untranslated region. *Numbering according to Tilley et al. (3).

The protocol was approved by the Ethics Committee of the School of Medical Sciences, UNICAMP. All blood samples were collected only after informed written consent was given. Information on ancestors of the study subjects was also obtained in order to characterize the ethnic background of both groups. Blood donors and patients were classified as "white" (Caucasian origin) and "non-white" (African descent). Among blood donors, 72% considered themselves white and 28% reported African ancestry. Ninety-one percent of the patients (N = 84) considered themselves white and only 9% (N = 8) had parents or grandparents of African ancestry. None of the individuals reported Amerindian or Asian ancestry in the two previous generations.

Genomic DNA for the PCR was isolated from blood leukocytes by standard methods (12). Four different primer sets were used to cover about 1.3 kb of the promoter region (Table 1) for DNA screening in the healthy individuals. Only the most conserved portion encompassing the Sp1-PBS and the two transcription initiation sites was analyzed in prostate cancer patients by amplification with two pairs of primers (1S/2AS and 3S/4AS) in separate reactions. PCR amplifications were performed in 50-µl reaction mixtures containing 200-500 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 20 pmol of each primer, 1X PCR buffer and 1 U Taq DNA polymerase (Gibco-BRL, Gaithersburg, MD, USA). After a first denaturation step (5 min at 94ºC), samples were subjected to 35 cycles of amplification at 94ºC for 90 s, annealing at 60-64ºC for 90 s and extension at 72ºC for 90 s, followed by a final extension step at 72ºC for 5 min. After amplification, PCR products were analyzed in ethidium bromide-stained agarose gels to confirm the predicted size of the fragments.

CSGE was performed on 10% acrylamide gel with 1.4-Bis (acryl oil) piperazine, 15% formamide and 10% ethylene glycol (13). Samples were prepared by mixing 5 µl of the PCR product from a previously sequenced control DNA with 5 µl of the PCR product of each corresponding fragment, and then incubated at 98ºC for 5 min, followed by 60-90 min at 68ºC. After this step, 5 µl of loading buffer (20% ethylene glycol, v/v, and 30% formamide, v/v, containing 0.025% xylene cyanol and 0.025% bromophenol blue) was added to each sample, which was then applied to the gel.

The CSGE was run at 300 V for 16 h at room temperature and stained with ethidium bromide. Sequence analysis was performed with the ThermoSequenase Radiolabeled Terminator cycle sequencing kit (Amersham, Cleveland, OH, USA) using radiolabeled 2',3'-dideoxynucleoside-5'-triphosphate nucleotide analogs (α-[^{33}]-ddNTPs). Sequencing was carried out twice with products from different PCR assays and with both sense and antisense primers to confirm the result.

Results and Discussion

CSGE band shift was evident in only one sample from an individual in the blood donor group, with the product of primers 3S/4AS. A T deletion (Figure 1) was located 24

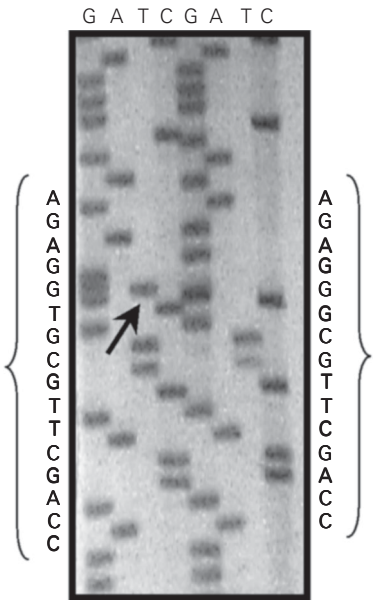

Figure 1. Sequencing analysis by conformation-sensitive gel electrophoresis of the mutation that caused the differential migration pattern. The four lanes on the left correspond to the normal promoter gene sequence. The four lanes on the right are from the blood donor's sample where the shift was detected. The arrow indicates the presence of the T base in the normal sequence.

nucleotides downstream from the TIS I (+36). No band shifts were detected in the samples from the prostate cancer patient samples.

The *AR* gene belongs to the nuclear receptor super-family and its functional domains have been well established and characterized. Several trinucleotide repeat sequences are found in the first exon of this gene, coding for the NH_2-terminal region required for transcriptional activation (14). One of them is a polymorphic glutamine repetition beginning at codon 58 in humans, and its expansion is associated with Kennedy's disease (15,16) while shorter CAG repeat lengths were found to correlate with an increased risk of aggressive prostate cancer (17).

Most of the molecular alterations detected in patients with prostate cancer are somatic mutations in coding regions, being distributed all over the *AR* gene (18). Of a total of 61 *AR* molecular alterations found in prostatic cancer tissue, only 4 are germ-line mutations (18). One of them, a germ-line mutation at codon 726 in exon 5 (R726L), was detected in two apparently unrelated Finnish prostate cancer patients (19). Another germ-line mutation is the glutamine to glutamic acid substitution at position 798 (Q798E) that occurs in exon 6 (20), which has also been described in two patients with partial androgen insensitivity syndrome (21,22) and in two individuals with azoospermia (23,24).

The other two germ-line mutations, characterized by Crocitto et al. (8), were located in the TIS II (G2T) and within one of the GC-rich regions of the 5'UTR (C214A). According to these investigators, these molecular alterations may contribute to the etiology of prostate cancer, changing the rates of gene transcription and/or translation.

In the present study, only one new mutation was detected, a T deletion at position +36, downstream from the TIS I (Figure 2). This mutation is located between the TIS and a GC-rich sequence within the same region where the two previously reported germ-line 5'UTR mutations were found (8). The deletion was detected in one healthy blood donor, a 45-year-old individual of Caucasian descent and father of three children. In this case, only *in vitro* expression and/or follow-up studies may permit us to determine whether this deletion compromises the *AR* gene function.

Considering that many genes are involved in prostate cancer etiology, we did not expect to find a large number of DNA sequence alterations in the *AR* promoter region of the prostate cancer patients. Indeed, the

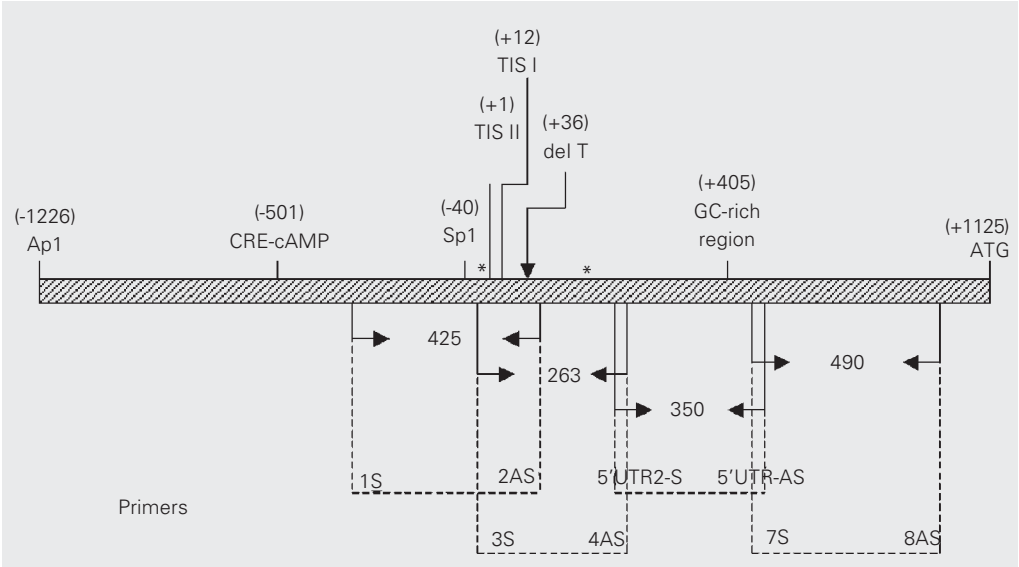

Figure 2. Schematic representation of the androgen receptor promoter region according to Tilley et al. (3). Asterisks indicate the mutation points described by Crocitto et al. (8). The vertical arrow shows the position of the T deletion detected in the present study. The lengths of PCR products (bp) are shown between arrows. Ap1 = Ap1 protein-binding site; AS = antisense; CRE-cAMP = cAMP-responsive element; S = sense; Sp1 = Sp1 protein-binding site; TIS = transcription initiation site; UTR = untranslated region.

fact that neither mutations nor SNPs were detected in these patient samples suggests that this sequence is strongly preserved towards a normal male phenotype (i.e., without androgen insensitivity) and that findings such as reported by Crocitto et al. (8) would be extremely rare. The possibility that some molecular alterations may be missing because CSGE may be not fully accurate in every situation needs to be taken into account. However, its sensitivity has been shown to reach 100% when an appropriate system of mildly denaturing solvents and moderate denaturation temperatures is employed (13,25) as has been done in the present study.

Sequence conservation in promoter regions is usually interpreted to be an indicator of functional importance (26). Up to now the promoter region of the *AR* gene was characterized only in humans (X78592, L14435), pigs (AF331845), mice (X59591), and rats (L15617) (27), proving to be conservative at the Sp1-PBS protein-binding site and in the TIS regions. These transcription-regulating elements are located from base -50 to +50 of the human HSARP sequence (numbering according to Tilley et al., 3), showing identity scores of 86, 88 and 94% when compared with mouse, rat and pig sequences (28), respectively.

In the present study, the finding of only one molecular alteration within the critical 5'UTR of the *AR* gene among 192 individuals reinforces the expected sequence conservation of this promoter region by vital selective pressure. Moreover, the absence of detectable variation in prostate cancer patients indicates a low probability of its involvement in prostate cancer etiology.

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