

Absolute measurement of androgen receptor mRNA in peripheral blood mononuclear, preputial skin and urethral mucosa cells of control individuals with phimosis using qRT-PCR

Valor absoluto do mRNA do receptor androgênico em células mononucleares do sangue periférico e células da pele do prepúcio e mucosa uretral em indivíduos controle com fimose, determinado por meio de qRT-PCR

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ABSTRACT

Introduction: Androgen actions are exerted upon the androgen receptor (AR), and complete genital virilization of normal 46,XY individuals depends on adequate function and expression of the AR gene in a tissue-specific manner. **Objective:** Standardization of normal ARmRNA in androgen-sensitive tissues. **Materials and methods:** In this study, we determined the quantitative amounts of ARmRNA in peripheral blood mononuclear, urethral mucosa and preputial skin cells of control subjects with phimosis by using RT-PCR. **Results:** The mean (SD) values of AR expression in blood, urethra and prepuce were: 0.01 (0.01); 0.43 (0.32); 0.31 (0.36), respectively. **Conclusion:** The AR expression is low in blood and equivalent in urethral mucosa and preputial skin, which may be useful in the diagnosis of individuals with abnormal external genitalia. Arq Bras Endocrinol Metab. 2011;55(8):665-8

Keywords

Androgen receptor; gene expression; genital tissue; phimosis

RESUMO

Introdução: As ações androgênicas são exercidas por meio do receptor androgênico (AR), e a completa virilização genital de indivíduos 46,XY normais depende de adequada expressão do gene AR de forma tecido específica. **Objetivo:** Padronizar valores normais de ARmRNA em tecidos sensíveis aos andrógenos. **Materiais e métodos:** Neste estudo, determinamos as quantidades de ARmRNA em células mononucleares do sangue periférico e em células da mucosa uretral e pele do prepúcio de indivíduos controles com fimose, utilizando RT-PCR. **Resultados:** A média (dp) dos valores de expressão do AR em sangue, uretra e prepúcio foram: 0,01 (0,01); 0,43 (0,32); 0,31 (0,36), respectivamente. **Conclusão:** A expressão do AR é baixa em sangue periférico e equivalente em mucosa uretral e pele prepucial, sendo sua quantificação útil no diagnóstico de indivíduos com alterações da genitália externa. Arq Bras Endocrinol Metab. 2011;55(8):665-8

Descritores

Receptor androgênico; expressão gênica; tecido genital; fimose

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INTRODUCTION

In 46,XY individuals, normal differentiation of male external genitalia is dependent on androgen effects in the androgen receptor (AR) (1). Adequate number and function of AR are needed in order to allow complete virilization of male genitalia.

Abnormal genital development can be determined by insufficient testosterone synthesis, inadequate testosterone conversion to dihydrotestosterone, or by decreased androgen action (2). At the AR level, in addition to AR gene mutations, both reduced amounts of AR mRNA or post-receptor abnormal response may be identified, resulting in a clinical condition classified as androgen insensitivity (3,4). Hypospadias are the most common clinical presentation of androgen insensitivity, presented as a spectrum of undermasculinized external genitalia (5).

The diagnosis of abnormal genitalia requires the knowledge of AR gene sequence and the recognition of AR gene expression. As a rule, gene expression is a tissue-specific phenomenon (6), in a way that it is important to standardize normal expected AR gene expression in each of the androgen-sensitive tissues.

Therefore, defining normal genital expression of the AR gene is key information to ensure precise diagnosis of 46,XY patients with abnormal external genitalia, including hypospadias or micropenises.

In this study, in order to establish normal reference values, we determined androgen receptor mRNA in peripheral blood mononuclear cells, and preputial skin and urethral mucosa cells of normal control individuals with phimosis, using quantitative RT-PCR.

PATIENTS AND METHODS

We studied 30 control individuals with phimosis grades I to III, and urethras with normotopic opening. Patients were in the prepubertal stage, with chronological age varying from 1.5 to 11.9 years. All individuals presented normotopic testes, with a testicular volume between 1 and 2 mL. The exclusion criteria were systemic or local treatment with steroids during the six months preceding surgery, and known gonadal dysgenesis or chromosomal abnormalities. The protocol was approved by the Ethics Committee of the institution, and parents or guardians signed a written consent form for patient inclusion in the study protocol.

Peripheral blood mononuclear cells, preputial skin and urethral mucosa were collected in order to obtain

RNA samples from blood and external genital tissues. This procedure was performed under anesthesia, during surgical postectomy. After separation with Histopaque-10771 (Sigma, Saint Louis, MO, USA) mononuclear cells were stored at -80°C with 10% DMSO (Sigma, Saint Louis, MO, USA). Preputial skin was removed from the dorsal region of prepuce-balanic transition and maintained at -80°C in RNAlater RNA stabilization reagent (QIAGEN GmbH, Hilden, Germany). Urethral mucosa cells were collected with a sterile swab (CB Products, Sao Paulo, Brazil), immediately placed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and also stored at -80°C . After no more than two weeks after sample storage, RNA of preputial skin was extracted employing the RNeasy Mini Kit (QIAGEN, GmbH, Hilden, Germany). RNA from blood and urethral mucosa cells was extracted using a phenol-chloroform protocol. Complementary DNA (cDNA) was obtained by reverse transcription, employing the TaqMan RT reagent kit (Applied Biosystems, Foster City, CA, USA). In each tissue sample, absolute ARmRNA was determined by quantitative Real Time PCR (qRT-PCR), employing FAM-labeled TaqMan probes, and the Applied Biosystems 7500 RT-PCR thermocycler. Primers and probe sequences to detect the AR gene have already been published. The TaqMan[®] MGB probe FAM[™] dye-labeled Assay ID (Hs00171172_m1) is based on RefSeq (NM_000044-2), and the assay location 3289 is in the boundary of exons 4-5, generating a 72-bp amplicon. (<https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=ABGEResults>).

In order to normalize the amount of RNA employed in each assay, AR values were corrected for the expression of a constitutive gene breakpoint cluster region (BCR). Primers and probe used for BCR detection have been previously described (Melo and cols., 2004). BCR sense primer: CCTTCGACGTCAATAACAAGGAT; BCR antisense primer: CCTGCGATGGCGTTCAC, and BCR probe: 6-FAM-TCCATCTCGCTCATCATCACCACGACA-TAMRA. Real time PCR conditions were: initial TaqGold activation at 95°C for 10 minutes, followed by product amplification in 45 cycles of 95°C / 15 seconds, and 60°C / 90 seconds.

All samples of each individual were tested in duplicate, in the same experiment, against the standard curves of both AR and BCR genes constructed with cDNA obtained from normal prostate gland of one cadaver donor. The final results of gene expression are shown as AR/BCR quantitative ratio.

The laboratory phase of the study was performed in 12 qRT-PCR experiments. In each experiment, the same standard curve based on normal prostate tissue was repeated in duplicate for at least three concentrations of cDNA (10^4 ; 10^3 ; 10^2 equivalent of cells). The coefficient of variation of CTs ranged from 5% to 7%. The percent variation between duplicates ranged from 2% to 4%.

Statistical analysis was carried out in SigmaStat for Windows v3.5 (SPSS, Chicago, IL, USA). Comparison between tissue samples from the same individual was done by non-parametric Friedman ANOVA for ranks. Values of $p < 0.05$ were considered significant.

RESULTS

We studied 30 control individuals with phimosis, with chronological age varying from 1.5 to 11.9 years. Clinical features are described in table 1. Penis size correlated significantly with age ($R = 0.66$, $p = 0.02$).

Quantitative results of AR mRNA are described in relation to the amount of constitutive gene BCR, and presented as AR/BCR ratio (Table 2 and Figure 1). AR expression was similar when urethral mucosa was compared with preputial skin. The expression of the AR gene was significantly higher in both genital tissues (preputial skin and urethral mucosa) in comparison with the expression of peripheral blood mononuclear cells (Friedman ANOVA). There was no correlation between age or penis size and AR expression in the three analyzed tissues.

Table 1. Clinical features of 30 control patients with phimosis

	Age (years)	Penis Size (cm)
Mean	4.8	5.3
SD	2.5	0.9
Median	4.3	5.3
p25 – p75	2.9 – 5.8	4.8 – 5.8
Range	1.5 – 11.9	3.8 – 7.9

Table 2. ARmRNA quantitative determination in peripheral blood mononuclear cells (PBMC), and urethral mucosa and preputial skin cells of patients with phimosis

	PBMC	Urethral mucosa	Preputial skin
Mean	0.01	0.43	0.31
SD	0.01	0.32	0.36
Median	0.003	0.37*	0.20*
p25 – p75	0.002 – 0.015	0.18 – 0.61	0.06 – 0.38
Range	0.0002 – 0.05	0.02 – 1.33	0.02 – 1.61

* Urethral mucosa or preputial skin vs. PBMC (Friedman ANOVA, $p < 0.05$). No difference was observed between urethral mucosa and preputial skin.

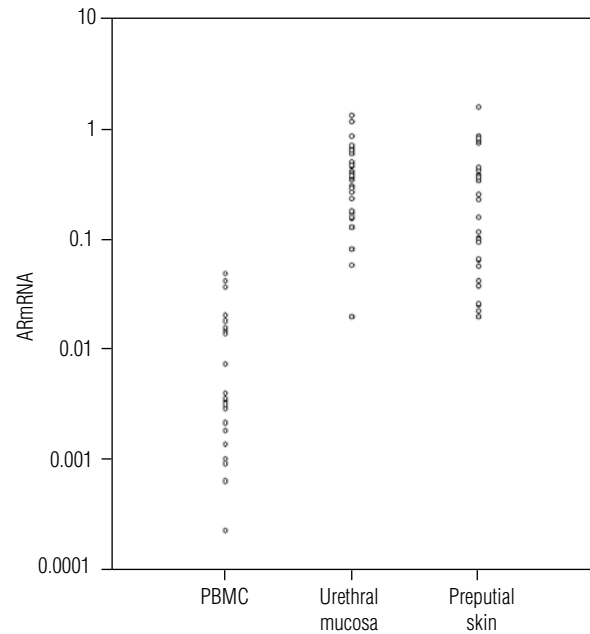


Figure 1. ARmRNA in peripheral blood mononuclear cells (PBMC), and in cells of urethral mucosa and preputial skin of patients with phimosis.

DISCUSSION

External genital differentiation and development is dependent on androgen sensitivity, and it is related to the action of testosterone and/or dihydrotestosterone on the androgen receptor. Abnormal androgen sensitivity has been recognized in patients with genital anomalies, such as cryptorchidism, micropenis and hypospadias. In these conditions, mutations in the AR gene are not always detected, and a reduction in AR mRNA has been considered potentially involved. In order to confirm this reduction, it is essential to standardize normal values of ARmRNA in a tissue-specific manner.

Direct quantitation of genital tissue ARmRNA is not available in the literature. In this study, the use of an absolute measurement of AR was possible by employing real time PCR. This method has proved to be reproducible and accurate, enabling AR measurement in the analyzed tissues.

Individuals with isolated phimosis, with no other genital anomaly, represented a control group. This group enabled the establishment of reference values for AR gene expression in peripheral blood mononuclear cells and cells from genital tissues. Our results demonstrated lower AR expression in peripheral blood mononuclear cells, when compared with the urethra and the prepuce. Therefore, ARmRNA determined in PBMC does not reflect the quantity of ARmRNA present in

genital tissues, and should not be used in the diagnosis of genital anomalies. Specific target-tissue sample is needed to recognize the androgen action. Preputial skin is the most prominent tissue related to genital differentiation and development, but presents limited availability in patients who do not need surgery. In this study, we also measured ARmRNA from genital cells obtained by a simple swab of the urethral mucosa. Our results demonstrated that these cells presented AR expression comparable to that observed in preputial skin. Swabbing urethral mucosa is a feasible procedure to obtain a representative tissue sample, and seems to be an adequate alternative to genital skin.

We concluded that the method we employed for absolute quantitation of ARmRNA is reproducible and enabled precise determination of gene expression in several tissues, including samples from external genitalia. AR expression in peripheral blood mononuclear cells is low, and not representative of genital androgen sensitivity. We also concluded that cells sampled from the urethral mucosa may be used in place of genital skin in the determination of genital expression of ARmRNA. This method presents the potential to be employed in the investigation of several conditions in which there is a suspicion of abnormal quantitative AR gene expression, such as disorders of sexual differentiation, and

in understanding phenotype/genotype variability in hyperandrogenic syndromes, such as congenital adrenal hyperplasia.

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REFERENCES

1. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer*. 2001;1(1):34-45.
2. Melo KF, Mendonça BB, Billerbeck AE, Costa EM, Latronico AC, Arnhold IJ. Androgen insensitivity syndrome: clinical, hormonal and molecular analysis of 33 cases. *Arq Bras Endocrinol Metabol*. 2005;49(1):87-97.
3. Brinkmann A, Jenster G, Ris-Stalpers C, van der Korput H, Brüggewirth H, Boehmer A, et al. Molecular basis of androgen insensitivity. *Steroids*. 1996;61(4):172-5.
4. Guerra G Jr, Mello MP. Insensibilidade androgênica. In: Maciel-Guerra AT, Guerra G Jr, editors. *Menino ou menina? Distúrbios da diferenciação do sexo*. Segunda edição, Rio de Janeiro, Brasil: Editora Rubio, 2010. p. 251-62.
5. Pierik FH, Burdorf A, Nijman JM, de Muinck Keizer-Schrama SM, Juttman RE, Weber RF. A high hypospadias rate in The Netherlands. *Hum Reprod*. 2002;17(4):1112-5.
6. Hoppe U, Holterhus PM, Wunsch L, Jocham D, Drechsler T, Thiele S, et al. Tissue-specific transcription profiles of sex steroid biosynthesis enzymes and the androgen receptor. *J Mol Med (Berl)*. 2006;84(8):651-9.