

## New single nucleotide variation in the promoter region of androgen receptor (AR) gene in hypospadias patients

Nasim Borhani<sup>1,2</sup> Ph.D. Candidate., Marefat Ghaffari Novin<sup>1,2</sup> M.D., Ph.D., Mehdi Manoochehri<sup>1</sup> Ph.D. Candidate., Mohsen Rouzrokh<sup>3</sup> M.D., Bahram Kazemi<sup>1</sup> Ph.D., Ameneh Koochaki<sup>1</sup> M.Sc., Ahmad Hosseini<sup>1</sup> Ph.D., Reza Masteri Farahani<sup>2</sup> Ph.D., Mir Davood Omrani<sup>4</sup> Ph.D.

1. Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

2. Department of Cell Biology and Anatomy, School of Medicine Shahid Beheshti University of Medical Sciences, Tehran, Iran.

3. Department of Pediatric Surgery, Mofid Children's Hospital, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

4. Department of Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

### Corresponding Author:

Mir Davood Omrani, Department of Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Velenjak Ave., Chamran Highway, Tehran, Iran.

Email: davood\_omrani@sbmu.ac.ir

Tel: (+98) 2122718505

Received: 27 October 2013

Accepted: 28 January 2014

### Abstract

**Background:** Hypospadias is one of the most common congenital abnormalities in the male which is characterized by altered development of urethra, foreskin and ventral surface of the penis. Androgen receptor gene plays a critical role in the development of the male genital system by mediating the androgens effects.

**Objective:** In present study, we looked for new variations in androgen receptor promoter and screened its exon 1 for five single nucleotide polymorphisms (SNP) in healthy and hypospadias Iranian men.

**Materials and Methods:** In our study, at first DNA was extracted from patients (n=100) and controls (n=100) blood samples. Desired fragments of promoter and exon 1 were amplified using polymerase chain reaction. The promoter region was sequenced for the new variation and exon 1 screened for five SNPs (rs139767835, rs78686797, rs62636528, rs62636529, rs145326748) using restriction fragment length polymorphism technique.

**Results:** The results showed a new single nucleotide variation (C→T) at -480 of two patients' promoter region (2%). None of the mentioned SNPs were detected in patients and controls groups (0%).

**Conclusion:** This finding indicates that new single nucleotide polymorphism in androgen receptor promoter may have role in etiology of hypospadias and development of this anomaly.

**Key words:** Hypospadias, Androgen receptor gene, Promoter.

*This article extracted from Ph.D. thesis. (Nasim Borhani)*

## Introduction

Hypospadias is the most common congenital malformation of the male external genitalia in which the urethral meatus opens on the ventral side of the penis (1). It affects about three to eight in 1000 newborn males (2). The location of the urethral meatus can differ from the penile glans of the penis to the scrotal or perineal region. The glandular and penile types are more common and considered as isolated hypospadias, whereas scrotal and prineal types are sporadic and considered as severe hypospadias (3). The androgen-androgen receptor interaction is critical for male external genitalia formation (4).

Impaired signaling through the androgen receptor (AR) leads to failure of urethral fold formation and fusion and consequently leads to hypospadias (5). The AR gene is located on chromosome Xq11-12 and consists of eight exons, in which exon 1 encodes the transactivation domain that activates

transcription of several different AR-responsive downstream genes (6, 7). The results of some previous studies showed the reduced androgen receptor expression level and androgen-binding capacity in genital skin fibroblasts of hypospadias patients (8, 9). These results suggest that mutations of the promoter region and transactivation domain of the AR gene could hinder or decrease the transcription machinery affinity for binding toward regulatory elements, thereby reducing the total amount of AR gene expression in the genital system. Therefore, in the present study, exon1 was screened for five single nucleotide polymorphisms (SNP), and in addition, AR gene promoter region was scanned for new variations in hypospadias disease in Iranian population.

## Materials and methods

### Patients and controls

The patients in this analytical association study referred to the Mofid's children Hospital

(the second referral center in Iran, Tehran) between March 2012 to August 2012. The patients should have criteria including diagnosis by urologist and having no sign of other genetic or congenital disease or genital malformations; (the properties distribution of patients in our study are listed in table I).

The controls were selected from healthy males in Shahid Beheshti School of Medicine. The control samples should not have any sign of genetic disease, genital malformations or any addiction. Peripheral blood samples of hypospadiac patients (n=100) and control group (n=100) were taken using venoject tube containing EDTA (0.5 M).

### DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from peripheral blood of patients and controls using the whole blood extraction kit (Dr. Gen TLE, Takara, Japan). Nucleotides -756 to -366 of the AR 5' promoter region (based on Ensemble.org genome browser) were amplified using the primer pair: 5'TCTCCAAA GCCACTAGGCAG3' (sense) and 5'ACCGAA GAGGAAAGGGCAGCTC3' (antisense). Nucleotides +604 to +888 of the AR transactivation domain were amplified using the primer pair: 5'TCCAGAGCGTGCGCGAA GTG3' (sense) and 5'CCGACTGCGGCTGT GAAGGT3' (antisense). The 30 µl reaction PCR reactions contained 100 ng genomic DNA, 100 pmol mixed primer pair, distilled water and master mix (Ampliqon, Denmark).

### Restriction fragment length polymorphism (RFLP) analysis

For transactivation domain genotyping, PCR, RFLP and 8% polyacrylamide gel electrophoresis (PAGE) were used. The RFLP reaction condition was detailed and presented in Table II.

### Promoter region sequencing

For AR promoter region sequencing, PCR products were purified using DNA extraction kit (Fermentas, Lithuania) and then scanned for variations using direct sequencing (Applied Bio systems 3730/3730xl DNA Analyzers Sequencing, Bioneer, Korea).

### Ethical considerations

The informed consent was obtained from all adult participants (control groups) and from the parents or legal guardians of minors (patients). This study had been reviewed and approved by a certified Ethical Committee in Shahid Beheshti University of Medical Sciences.

## Results

### RFLP results

The 285 bp PCR product containing five SNP sites was digested using suitable enzymes; SsiI, PstI, CciI, SfaNI, and BseGI. The two SNPs rs62636528, and rs62636529 produce gain of sites compared to wild type allele, while rs78686797, rs139767835, and rs145326748 supposed to have loss of sites in digestion with the mentioned enzymes (Table II). The SsiI, PstI, and BseGI could cut wild type alleles and produce 2-3 shorter fragments that could be visualized on PAGE; while the CciI and SfaNI will cut mutant alleles and leave the intact wild types.

### Sequencing results

AR gene promoter region was scanned for new variations in subjected samples and a new single nucleotide polymorphism (SNP) was detected in two patient samples (2%) (One glandular and one distal penile type) at position -48°C→T (the normal sequence and two sequences curves with a new nucleotide variation are shown in Figure 1).

**Table I.** The properties distribution of patients. The different number of each hypospadias subtype, presence (yes) or absence (no) of Parental Consanguinity and Family history are depicted

Type	Patients number*	Parental consanguinity		Family history	
		Yes	No	Yes	No
Penoscrotal	13	1	12	1	12
Proximal penile	2	-	2	-	2
Mid shaft	7	2	5	-	7
Distal penile	53	8	45	5	48
(sub)Coronal	5	1	4	-	5
Glandular	20	2	18	1	19

\*Total patients number = 100

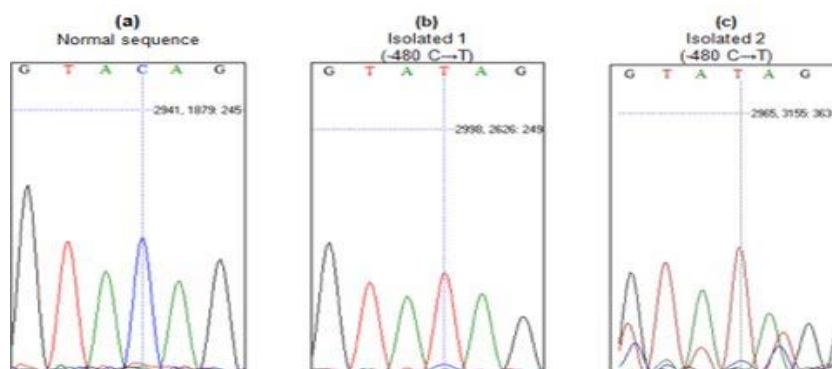
**Table II.** Restriction fragment length polymorphism protocol for exon 1 genotyping

SNP	Restriction enzyme	Gain of site	Loss of site
rs139767835	SsiI <sup>a</sup>		•
rs78686797	PstI <sup>a</sup>		•
rs62636528	CaiI <sup>a</sup>	•	
rs62636529	SfaNI <sup>b</sup>	•	
rs145326748	BseGI <sup>a</sup>		•

All reactions were prepared in order to the enzymes protocol.

<sup>a</sup>Fermentase (Ukraine)

<sup>b</sup>Biolabs (UK)



**Figure 1.** (a) Normal sequence from control group (C nucleotide in -480), (b) Isolated 1 from patient group (C→T in -480), (c) Isolated 2 from patients group (C→T in -480).

## Discussion

AR gene is a member of the nuclear receptor superfamily that is located on Xq11-12. This receptor plays a critical role in male sexual differentiation by mediating the biological effects of androgens (10). Early development of external genital system is similar for both sexes but urethral folds fusion and masculinization process is androgen-androgen receptor dependant phase. Therefore any changes in AR amount or structure may disrupt this patterning (11). In our knowledge, we scanned the promoter region of AR for the first time in hypospadiac patients and we found a new single nucleotide polymorphism in two patients at -480 using direct sequencing. There have been many reports on AR promoter sequencing.

In 2006, Waltering *et al* scanned AR gene promoter and untranslated region in 44 clinical prostate cancer specimens and 36 normal controls; but they did not find any different sequence variations (12). Likewise, Ghadessy *et al* screened promoter region of androgen receptor gene in 240 men with idiopathic infertility and 30 healthy men and this study did not show presence of any deletions or mutations in infertile or healthy men and they suggested that AR gene promoter mutation are not common in men with idiopathic infertility (13). Furthermore a study on AR gene promoter region of 100 healthy men suggested that DNA sequence alterations are

rare in the human androgen receptor gene promoter (14).

Additionally, we did not find any of mentioned single nucleotide polymorphisms in control and patient groups using PCR and RFLP techniques. This finding may declare that these five polymorphisms are not be involved in hypospadias etiology. These data are consistent with the previous report by Muroya *et al* in which mutation screening of genomic DNA of men with hypospadias did not show any point mutation in AR gene exons (3). Also Hiort *et al* reported that the majority of patients with hypospadias did not carry androgen receptor gene mutations. In addition Sasagawa *et al* suggested that alteration of the AR gene is rare in males with isolated hypospadias, cryptorchidism, micropenis, or idiopathic male infertility (5). Furthermore, Radpour *et al* reported that none of the studied hypospadiac patients in their research had AR gene mutations (15). Likewise, other scientists suggested that AR gene mutation is rare in hypospadiac patient (16, 17).

In addition, In a recent study by Adamovic *et al* it was found that a SNP in the AR gene region was associated with susceptibility to severe hypospadias. They concluded that AR rs5919436 polymorphism may act as a new gene marker for increased susceptibility to severe hypospadias in Caucasians (18). Besides, another study has investigated the possible association of the CAG repeat

length in the *AR* gene with the hypospadias; and suggested that expanded CAG repeat length has a role in modifying the risk and development of hypospadias (19).

In addition, in another study on Chilean population, the CAG and GGN polymorphisms in the *AR* gene has been investigated and showed that in isolated hypospadias boys, there are longer CAG alleles in their *AR* gene that might be associated with the development of hypospadias (20). Taken together, our result suggests that a same nucleotide variation in *AR* gene promoter of 2 patients with isolated hypospadias may have role in etiology of this congenital abnormality in mentioned patients by reducing the total amount of *AR* gene expression in the genital system due to decreasing the transcription machinery affinity for binding toward regulatory elements.

### Acknowledgments

This study was supported by a grant from the Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant number: 8562). The authors would like to thank Dr. Mojgan Bandehpour for technical consulting and Farnaz Safavi for providing us with the patient's blood samples.

### Conflict of interest

The authors declare that there is no conflict of interest regarding the study.

### References

1. Carmichael SL, Shaw GM, Lammer EJ. Environmental and genetic contributors to hypospadias: a review of the epidemiologic evidence. *Birth Defects Res A Clin Mol Teratol* 2012; 94: 499-510.
2. Carmichael SL, Shaw GM, Nelson V, Selvin S, Torfs CP, Curry CJ. Hypospadias in California: trends and descriptive epidemiology. *Epidemiology* 2003; 14: 701-706.
3. Muroya K, Sasagawa I, Suzuki Y, Nakada T, Ishii T, Ogata T. Hypospadias and the androgen receptor gene: mutation screening and CAG repeat length analysis. *Mol Hum Reprod* 2001; 7: 409-413.
4. Baskin LS. Hypospadias and urethral development. *J Urol* 2000; 163: 951-956.
5. Sasagawa I, Suzuki Y, Muroya K, Ogata T. Androgen receptor gene and male genital anomaly. *Arch Androl* 2002; 48: 461-466.
6. Jaaskelainen J, Mongan NP, Harland S, Hughes IA. Five novel androgen receptor gene mutations associated with complete androgen insensitivity syndrome. *Hum Mutat* 2006; 27: 291.
7. Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* 1994; 22: 3181-3186.
8. Svensson J, Snochowski M. Androgen receptor levels in preputial skin from boys with hypospadias. *J Clin Endocrinol Metab* 1979; 49: 340-345.
9. Keenan BS, McNeel RL, Gonzales ET. Abnormality of intracellular 5 alpha-dihydrotestosterone binding in simple hypospadias: studies on equilibrium steroid binding in sonicates of genital skin fibroblasts. *Pediatr Res* 1984; 18: 216-220.
10. Simon S, Mueller SO. Human reporter gene assays: transcriptional activity of the androgen receptor is modulated by the cellular environment and promoter context. *Toxicology* 2006; 220: 90-103.
11. van der Zanden LF, van Rooij IA, Feitz WF, Franke B, Knoers NV, Roeleveld N. Aetiology of hypospadias: a systematic review of genes and environment. *Hum Reprod Update* 2012; 18: 260-283.
12. Waltering KK, Wallen MJ, Tammela TLJ, Vessella RL, Visakorpi T. Mutation screening of the androgen receptor promoter and untranslated regions in prostate cancer. *Prostate* 2006; 66: 1585-1591.
13. Ghadessy FJ, Liow SL, Yong EL. Mutations in the promoter region of the androgen receptor gene are not common in males with idiopathic infertility. *Mol Hum Reprod* 1999; 5: 287-290.
14. Cabral DF, Santos A, Ribeiro ML, Mesquita JC, Carvalho-Salles AB, Hackel C. Rarity of DNA sequence alterations in the promoter region of the human androgen receptor gene. *Braz J Med Biol Res* 2004; 37: 1789-1794.
15. Radpour R, Rezaee M, Tavasoly A, Solati S, Saleki A. Association of long polyglycine tracts (GGN repeats) in exon 1 of the androgen receptor gene with cryptorchidism and penile hypospadias in Iranian patients. *J Androl* 2007; 28: 164-169.
16. Allera A, Herbst MA, Griffin JE, Wilson JD, Schweikert HU, McPhaul MJ. Mutations of the androgen receptor coding sequence are infrequent in patients with isolated hypospadias. *J Clin Endocrinol Metab* 1995; 80: 2697-2699.
17. Sutherland RW, Wiener JS, Hicks JP, Marcelli M, Gonzales ET Jr, Roth DR, et al. Androgen receptor gene mutations are rarely associated with isolated penile hypospadias. *J Urol* 1996; 156: 828-831.
18. Adamovic T, Thai HT, Lieden A, Nordenskjold A. Association of a tagging single nucleotide polymorphism in the androgen receptor gene region with susceptibility to severe hypospadias in a caucasian population. *Sex Dev* 2013; 7: 173-179.
19. Adamovic T, Nordenskjold A. The CAG repeat polymorphism in the androgen receptor gene modifies the risk for hypospadias in Caucasians. *BMC Med Genet* 2012; 13: 109.
20. Parada-Bustamante A, Lardone MC, Madariaga M, Johnson MC, Codner E, Cassorla F, et al. Androgen receptor CAG and GGN polymorphisms in boys with isolated hypospadias. *J Pediatr Endocrinol Metab* 2012; 25: 157-162.