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ORIGINAL ARTICLE

Association of *SRD5A2* gene mutations with risk of hypospadias in the Iranian population

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Abstract
Background Hypospadias is one of the most common forms of congenital malformation of the male external genitalia worldwide. The ratio in the Iranian population is one in 250 live male births. The conversion of testosterone to dihydrotestosterone (DHT) in the presence of steroid 5α-reductase 2, which is encoded by *SRD5A2* gene, plays an important role in the normal development of the male reproductive system.
Methods We examined whether *SRD5A2* gene mutations (V89L and A49T polymorphisms) are associated with the risk of hypospadias in the Iranian population. We performed exons sequencing for *SRD5A2* gene in 109 hypospadias patients.
Results We identified two new mutations in the subgroups of affected cases: including a substitution of the nucleotide T > A in the codon 73 [c.219T > A (p.Leu73_Ser74insHisPro)] and an insertion of an extra A nucleotide in the codon 77 [c.229insA* (p.Gly77*)]. Additionally, we performed PCR–RFLP for the two identified polymorphisms and revealed that V89L [OR = 5.8, 95% CI (3.8–8.8), p value < 0.001] and A49T [OR = 10.16, 95% CI (3.94–26.25), p value < 0.001] are significantly associated with hypospadias occurrence in patients. Our haplotype analysis further indicated that the Leu–Ala haplotype increases risk of hypospadias; conversely, the Val–Ala haplotype decreases the risk of hypospadias in the studied patients.
Conclusions This study demonstrates that polymorphisms in the *SRD5A2* gene could be considered as a risk factor for hypospadias disease emergence.

Keywords Hypospadias · *SRD5A2* gene · Mutation · Iranian population

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Introduction

Hypospadias is the second most common congenital malformation of the male external genitalia after cryptorchidism [1], affecting one in 200–300 live male births [2]. Hypospadias disease is often associated with a penile curvature (Chordee) [3]. Classification of hypospadias phenotypes depends on the severity of the anatomical displacement of the urethral meatus [4, 5]. This disease is classified into three groups including mild, moderate and severe. The mild phenotype is the distal form in which the urethra opens on the anterior body of the penis (glandular,

subcoronal). The moderate phenotype refers to defects in the middle portion of the penis (distal penile, mid shaft, proximal penile). The severe phenotype is the proximal type which involves posterior penile (penoscrotal, scrotal, perineal) [5].

Normal development of male reproductive tissue, including the urethra, requires testosterone and dihydrotestosterone (DHT) [6]. Testosterone is converted to the more potent androgen, DHT, by an enzyme known as asteroid 5α-reductase 2. This enzyme is encoded by the *SRD5A2* gene (OMIM: 607306) that is located on chromosome 2p23 [7, 8]. DHT deficiency affects the normal differentiation of male external genitalia, as well as the maturity of male secondary sexual traits during puberty [9]; and the DHT synthesis is variably impaired, depending on the residual activity of the mutated protein [10]. Previous studies have shown that mutations in *SRD5A2* may lead to a reduction of its enzyme activity and result in various degrees of masculinization defects, including hypospadias [11–15].

Recently, Dung and colleagues have reported the novel missense mutation (c.659 C > T; pS220L) in the patients with 46,XY disorder of sex development (DSD) [16]. Moreover, the IVS1-2A > G splice mutation has been reported in the Cypriot patients to cause 5α-reductase deficiency [17]. Furthermore, two polymorphisms in *SRD5A2,* including V89L (a substitution of valine by leucine at codon 89 due to a G > C transversion, rs523349) [18] and A49T (substitution of alanine by threonine at codon 49 due to a G to A transition, rs9282858), have been shown to be associated with hypospadias in different populations [19–21]. In the present study, we sought to identify whether the specific genotypes of *SRD5A2* are related with the risk of hypospadias in the Iranian population.

Materials and methods

Experimental subjects

Hundred and nine blood samples were collected from patients with hypospadias at Mofid Children's Hospital. The criteria for selecting participants in the study included diagnosis by urologist, and having no sign of other genetic or congenital disease or genital malformations, the characteristic of patients in our study are depicted in Table S1. These patients were categorized according to the phenotypes of hypospadias and the presence of Chordee. A total of 109 matched controls from the Shahid Beheshti School of Medicine hospital without hypospadias or any history of genital abnormalities who visited the urologist for some other reasons were collected via person to person surveys and were confirmed by a urologist. Demographic and clinical data pertaining to the reproductive profile of each participant and their and family, as well as genital abnormalities, were recorded for cases and controls. Peripheral blood samples of hypospadic patient and control groups were taken using Venoject tubes containing EDTA (0.5 M). DNA samples were extracted using DNA extraction kits according to the manufacturer's protocol (Qiagen,Valencia, California).

Mutation analysis

The genomic DNA was amplified for the exons of SRD5A2 gene by PCR. Using sequencing primers, the PCR products were sequenced directly with the Macrogene DNA Sequencer (Company). The variations of normal sequence obtained through applying NCBI-BLAST and Ensembl data base. The PCR products from cases and controls were subsequently sequenced again to confirm the original results.

In silico functional analysis of L73H variant

To assess the potential functional impact of the L73H (T > A substitution in codon 73), we utilized I-Mutant v.2 algorithms to perform in silico analysis. If a defined 3D (three dimensional) structure of a given protein was not available, comparative homology modeling might be the most accurate method of choice to generate the reliable tertiary protein structure using sequence information. We used the I-TASSER program [22] to generate the *SRD5A2* tertiary structure and then used the PyMOL (https://www.pymol.org/) to visualize *SRD5A2* products in both wild type and mutant forms. The HOPE [23] was used for more evaluation of p.L73H on the *SRD5A2* product structure. The combination of the two predictive value tools, sorting intolerant from tolerant (SIFT) and polymorphism phenotyping (PolyPhen), has high accuracy on predicting pathogenicity of novel mutations [24, 25]; in this case, we used both tools.

Genotyping of V89L and A49T polymorphisms

The *SRD5A2* gene was analyzed for V89L and A49T polymorphisms using restriction fragment length polymorphism (RFLP) [26]. The PCR products for the nominated polymorphisms were digested with two restriction endonucleases, *Rsa*I and *Mwo*I, respectively (Fig. S1). Then PCR products were electrophoresed on 14% polyacrylamide gel according to the manufacturer's protocol.

Statistical analysis

The genotype and haplotype frequencies, also D' value between possible pairing of V89L and A49T polymorphisms, were calculated using GENEPOP (http://genepop.curtin.edu.au/), PHASE and 2LD programs, respectively [27, 28]. The association of the hypospadias risk with V89L and A49T was verified by case–control genetic association analysis (http://www.oege.org/software/orcalc.html) and MedCalc software (http://www.medcalc.org/calc/odds_ratio.php). The most frequent homozygote genotypes in controls were used as reference values. Pearson Chi-square test was employed to calculate the deviation from Hardy–Weinberg equilibrium (HWE); p values less than 0.05 were considered in the H–W equilibrium.

Results

SRD5A2 sequencing results

We found three mutations consist of two new mutations and one previously identified mutation in cases. The clinical description of these patients is shown in Tables S1, S2. The two new mutations were located in the exon one consist of a T > A substitution in codon 73 (L73H; Fig. S2), and one nucleotide (A) that was inserted at nucleotide position 229 in codon77 [229insA] of the *SRD5A2* gene (Fig. S3). In addition, we identified the previously reported substitution of the nucleotide G > A in codon 196 (G196S) in the exon 4 of *SRD5A2* in one of the patients (Fig. S4).

Additionally, our sequencing results for four patients showed changes in the exon five of the *SRD5A2* gene as compared with a normal sequence. Three patients had with T > A replacement in the second nucleotide of codon 256 (rs28383083; Fig. S5). Also we found three polymorphisms, rs192604242, rs72040241 and rs10529926, in one of the patients (Fig. S6).

Bioinformatics analysis

In silico analysis of the [229insA] in exon 1 showed an altered the open reading frame of the *SRD5A2* gene, causing a different translation from the normal protein. In addition, the sequence translation indicated that this frame shift mutation creates an early stop codon, resulting in a truncated protein. Furthermore, we performed in silico analysis to predict functional/structural deficits conferred by the L73H mutation in *SRD5A2*. The 3-oxo-5-alpha-steroid 4-dehydrogenase 2 is a transmembrane protein (254 aa) that plays a central role in sexual differentiation in the androgen receptor pathway. I-Mutant V.2 showed that p.L73H slightly decreases protein stability. We used I-TASSER to perform homology modeling and obtained a tertiary structure of *SRD5A2* product (Fig. S7). However, the analysis demonstrated that the mutated residue is located in a domain that is important for the main activity of the protein. Mutation of the residue might not significantly disturb this function (Table S3). Moreover, the prediction of SIFT showed that L73H has non-deleterious (neutral) function in the protein; additionally, PolyPhen shows that this is a benign substitution which does not affect the three-dimensional structures of protein (Table S3).

A49T and V89L polymorphisms

Phenotype data and frequency distribution of cases are classified as shown in Table S1. The phenotypic subgroups including glandular, midshaft and penoscrotal were observed with the most common frequencies within mild, moderate and severe phenotypes, respectively. Approximately, half of the hypospadias cases ($n = 52$) showed the moderate phenotype with 33% Chordee frequency (Table S1).

We found significant associations of V89L [OR 5.8; 95% CI 3.8–8.8; p 0.001 < 0.05] and A49T [OR 10.16; 95% CI 3.94–26.25; p 0.001 < 0.05] with the risk of hypospadias in our population (Table 1). The results of A49T genotype analysis revealed a high frequency of (AA) in both case and control groups, even though we found only five persons with (AT) genotype in the control group. Moreover, we did not detect the TT genotype in any control group. The less common allele (T) frequency in the control and case was (0.02) and (0.19), respectively (Table 1). *SRD5A2*-V89L analysis showed that the most frequent genotype between case and patient was (VL). In addition, the majority of patients had (LL) and controls had (VV) genotypes. The most frequency allele in controls and patients was V (0.63) and L (0.77), respectively (Table 1). We performed a Hardy–Weinberg equilibrium (HWE) test for both studied polymorphisms (V89L and A49T) in the *SRD5A2* gene where the genotypic distributions of these polymorphisms showed a p value less than 0.05. However, the A49T polymorphism revealed a significant p value in hypospadias patients, indicating a deviation from (HWE; Table 1). Also, according to the classification of hypospadias phenotypes, the more severe clinical features, 64% of the severe group had AT genotype.

According to phenotypic severity, although the genotypic distribution varied between three mild, moderate and severe groups; the V89L and A49T polymorphisms showed the significant association with each category (p value < 0.05). Highest frequency of LL genotype of V89L was in the moderate group of cases; also AT genotype was the most frequent genotype in severe groups (Table 2).

Table 1 Distribution of SRD5A2 genotypes and the allele frequencies along with their associations with the risk of hypospadias

Polymorphism	Population (*N*)	*SRD5A2* genotype			Allele frequency		*p* value (HWE)	Effect size (95% CI)	*p* value (association)
		AA	AT	TT	A	T			
A49T	Control (109)	104	5	0	0.98	0.02	0.98	10.16 (3.94–26.25)	<0.001
	Patient (109)	67	42	0	0.81	0.19	0.049		
	VV	VL	LL	V	L				
V89L	Control (109)	48	42	19	0.63	0.37	0.22	5.8 (3.8–8.8)	<0.001
	Patient (109)	9	32	68	0.23	0.77	0.19		
Polymorphism	Population (*N*)	*SRD5A2* genotype			Allele frequency		*p* value (HWE)	Effect size (95% CI)	*p* value (association)
		VV	VL	LL	V	L			
V89L	Control (109)	48	42	19	0.63	0.37	0.22	5.8 (3.8–8.8)	<0.001
	Patient (109)	9	32	68	0.23	0.77	0.19		

SNPs genotype frequencies were examined for HWE by using the Chi-square statistic Graph Pad software, and all were found out to be consistent ($p < 0.05$) with HWE among Iranian controls. Data were analyzed by using unconditional logistic regression to calculate an odd ratio (OR) as an estimate of relative risk of hypospadias associated with V89L and A49T genotypes

N Number of individuals analyzed, *HWE* Hardy–Weinberg

Table 2 Association of genotypes with phenotypes in hypospadias patients

Groups	GenotypeV89L count (%)			Genotype A49T count (%)	
	VV	VL	LL	AA	AT
Mild	4 (12.5%)	8 (25%)	20 (62.5%)	29 (90.6%)	3 (9.4%)
Moderate	3 (5.77%)	10 (19.23%)	39 (75%)	31 (59.6%)	21 (40.4%)
Severe	2 (8%)	14 (56%)	9 (36%)	9 (36%)	16 (64%)
Total	9 (8.3%)	32 (29.4%)	68 (62.4%)	69 (63.3%)	40 (36.7%)

Patients are phenotypically grouped with their respective genotypes. The V89L and A49T polymorphisms showed the significant association with each category (p value < 0.05). Highest frequency of LL genotype of V89L was in the moderate group of cases; also AT genotype was the most frequent genotype in severe groups

In haplotype verification, the control group haplotypes L–T, L–A and V–A indicate informative haplotypes in the studied population. We did not detect haplotype V–T in the control group, implying a rare haplotype in the healthy ones (Table 3). Among all four possible haplotypes, the highest frequency belongs to L–A haplotype in patients (Table 3). As shown by the LD analysis in Table 3, the D' value of pairing marker A49T-V89L was >0.3 in the hypospadias patients and controls, consistent with the χ^2 value estimated for this pairing of markers (p value < 0.05). These results support linkage disequilibrium between the two polymorphisms *SRD5A2*-A49T and *SRD5A2*-V89L in our population.

Discussion

In the present study, we investigated the association between polymorphisms in *SRD5A2* gene and hypospadias disease in the Iranian population. We are the first to report mutations on the exon one of *SRD5A2* gene. Our in silico analysis suggested that these mutations may change the structure and function of this gene. The newly identified L73H mutation leads to a substitution change from

Table 3 Haplotype frequency and linkage disequilibrium analysis of *SRD5A2* polymorphisms

Population	Haplotype frequency				LD analysis		
	V–A	L–A	L–T	V–T	D'	X^2	*p* value
Control	0.55	0.34	0.12	0	1	10.64	0.001
Patient	0.17	0.66	0.11	0.07	0.36	19.8	0.0009

We performed haplotype and linkage disequilibrium analysis for the SRD5A2 SNPs test. Among all four possible haplotypes, the highest frequency belongs to L–A haplotype in patients, whereas the control group haplotypes L–T, L–A and V–A indicate informative haplotypes in the studied population; we did not detect haplotype V–T in the control group, implying a rare haplotype in the healthy ones

CTC (Leucine) to CAC (Histidine) that does not affect the enzyme function. The other new mutation [229insA] causes a truncated protein which could be deleterious, whereas the new mutations, C.229insA (G77 fs) and C.219T > A (L73H), have not been previously reported. Further research is required to determine the biological role of these mutations and the extent to which they affect enzyme activity. In addition, we identified the mutation G196S in one of our hypospadias patients which is located in the exon 4 of *SRD5A2*. This mutation has also been reported in previous studies [21, 29, 30].

The sequencing results suggested that the exons 1 and 4 might be hotspots for *SRD5A2* mutations in Iranian hypospadias patients. We found a strong association between the A49T variant and risk of hypospadias in our population. The allele *SRD5A2*-A49 was the highest frequency in both patient and control groups. Previous studies have indicated that A49T mutation in *SRD5A2* could be considered a risk factor for hypospadias, by reducing the level of androgens [20, 31]. Our results also showed a deviation from Hardy–Weinberg equilibrium for this polymorphism in our patients. This might be a result of TT genotype's inconsistency with health and survival which was not even detected in previously studied groups. For instance, Pearce CL et al. could not find a TT genotype in the 1200 case and control samples [32]. Similarly, Jun Li and colleagues indicated that they observed less than 0.01% frequency of TT genotype [33].

The association between *SRD5A2*-V89L polymorphism and hypospadias patients has been studied in different populations [19, 21, 34]. Here, we have confirmed that the allele and genotype frequency of V89L polymorphism are different in case versus control. The *SRD5A2*-89L allele was more common in heterozygous and homozygous forms in the patient group. The most observed phenotype in patients, the moderate phenotype (midshaft), was associated with LL genotype of *SRD5A2*-V89L polymorphism.

Our results from the allele frequency estimation further suggested that the allele *SRD5A2*-V89 might have a protective role for hypospadias. In contrast, the allele *SRD5A2*-89L appears to increase the risk of hypospadias in the Iranian population. Our results therefore indicate a correlation of *SRD5A2*-V89L and *SRD5A2*-A49T with hypospadias disease in the Iranian population.

We also showed that L–A and V–A are more common haplotypes in both patient and control groups. These results may indicate that L–A haplotype confers a significant risk of hypospadias in Iranian patients. In contrast, our results indicated that V–A haplotype is associated with protection against this disease.

In conclusion, our findings suggest that two polymorphisms of *SRD5A2*, V89L and A49T, may harbor susceptibility to hypospadias disease. In addition to the importance of polymorphisms in this gene, *SRD5A2* mutations cause in incomplete masculinization of male external genitalia and sex development; these phenotypes can range from almost normal female structures to a distinct male phenotype with ambiguous genitalia at birth. These phenotypes result from impaired conversion of testosterone to dihydrotestosterone (DHT) due to mutations in the *SRD5A2* gene. Further studies in other population incorporating the DHT levels of patients and in vitro experiments (cell culture and animal studies) will provide additional insight into biological mechanisms which underlie the role of *SRD5A2* and its polymorphisms in hypospadias.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent This informed consent form collected from parents of children between 1 and 4 years of age who attended to Mofid Children's Hospital, and who were asking to participate in the study.

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