The p.G146A and p.P125P Polymorphisms in the Steroidogenic Factor-1 (*SF-1*) Gene Do Not Affect the Risk for Hypospadias in Caucasians

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Key Words

Hypospadias · Mutation analysis · *NR5A1* · SNP · *SF-1*

Abstract

Hypospadias is a frequent congenital malformation in boys and is characterized by incomplete fusion of the urethral folds. The steroidogenic factor-1 (*SF-1*, *NR5A1*) gene plays a key role in hypothalamic-pituitary-steroidogenic organ development, and has previously been reported to be mutated in individuals with 46,XY disorder of sex development. Here, we investigated the role of *SF-1* in hypospadias, a milder form of 46,XY disorder of sex development. We performed direct sequencing analysis of the *SF-1* gene in 2 male Caucasian twins exhibiting very severe hypospadias, and in 95 Caucasian boys with mild and severe hypospadias. We further extended the analysis by investigating 332 mild and severe hypospadias cases and 422 male controls using TaqMan assays. Our sequencing revealed a novel heterozygous p.R313H (c.938G>A) missense mutation in each twin, and no mutations in the 95 Caucasian cases. Instead, a missense p.G146A (c.437G>C), and a silent known p.P125P (c.375C>T) polymorphism, respectively, was found in several of the lat-

ter cases. Further investigation of the 2 polymorphisms in the larger material of cases and controls showed no significant genotypic or allelic association. In conclusion, the *SF-1* gene may not play a significant role in the development of hypospadias in Caucasians. Copyright © 2012 S. Karger AG, Basel

Hypospadias is characterized by incomplete fusion of the urethral folds in boys, and is one of the most common congenital malformations. The incidence of hypospadias in Sweden is 3 per 1,000 male births since the beginning of the 1970s according to the annual Swedish Malformation Registry (hypospadias rates worldwide are reviewed elsewhere [Fisch et al., 2010]). Furthermore, hypospadias is mainly a complex genetic disorder, and its development is most likely influenced by a combination of multiple genetic and environmental factors. During recent years, candidate genes such as the mastermind-like domain containing 1 (*MAMLD1*), activating transcription factor 3 (*ATF3*), zinc finger E-box-binding homeobox 1 (*ZEB1*) genes [Liu et al., 2005; Ogata et al., 2008; Qiao et al., 2011], among several others, have been implicated in the etiol-

ogy of hypospadias. Still, most cases of hypospadias lack a molecular diagnosis.

Normal penile and urethral development begins in the sixth week of gestation with the formation of the urogenital sinus on the genital tubercle. The 2 edges of the urethral groove fuse in a proximal-to-distal direction, forming the urethra. Finally, the prepuce originates from the periphery of the glans penis and is complete by week 14 of fetal gestation. Variable loss-of-function changes of transcription factors, receptors, and enzyme pathways involved in Leydig cell development and androgen biosynthesis, may induce incomplete fusion of the urethral folds, and cause hypospadias [Wilhelm and Koopman, 2006].

The steroidogenic factor 1 (*SF-1*) gene, also known as adrenal 4-binding protein (*AD4BP*) or nuclear receptor subfamily 5 group A number 1 (*NR5A1*), is located on the long arm of HSA9 (9q33) and consists of 7 exons. It codes for a 461 amino acid protein that shares structural homology with other members of the nuclear receptor superfamily [Wong et al., 1996]. This gene was initially suggested to play an important role in patients with primary adrenal failure, 46,XY gonadal dysgenesis and Müllerian structures, since similar phenotypes were detected in *SF-1* knock-out mice by 3 independent groups [Luo et al., 1994; Sadovsky et al., 1995; Shinoda et al., 1995]. However, with time, these combined clinical features turned out to be rare in humans. In addition, only 3 *SF-1* mutations were detected in these patients, suggesting a minor role of *SF-1* in the investigated disease phenotypes [Achermann et al., 1999, 2002; Biason-Lauber and Schoenle, 2000]. The role of *SF-1* in the embryo has further been reported to regulate the expression of enzymes involved in the steroidogenic system from week 8 of gestation, which results in masculinization of the external genitalia [Ikeda et al., 1994].

Furthermore, critical functional domains of *SF-1* include an amino-terminal 2 zinc finger DNA-binding domain, an accessory DNA-binding region, a hinge region, and a ligand-binding domain (LBD) that forms an AF-2 structure, which regulate the transcription of a vast array of genes involved in adrenal and gonadal development, sex differentiation, steroidogenesis and reproduction by the hypothalamic-pituitary-adrenal/gonad axis [Morohashi et al., 1992]. Thus, relatively small changes of the *SF-1* activity during the critical period in the embryo may cause a 'cascade reaction' in the endocrine system, which in turn may lead to clinically harmful effects.

Moreover, an increasing number of studies report variations and/or mutations in the *SF-1* gene that may be associated with the etiology of the 46,XY disorder of sex

development, severe hypospadias and other human reproductive phenotypes [Köhler et al., 2009]. In the present study, we intended to investigate the role of *SF-1* in the disease of hypospadias in a Caucasian population.

Materials and Methods

Direct Sequencing

Two monozygotic Caucasian male twins with very severe hypospadias, to such a degree that a gender assignment was difficult, and their parents, in addition to a total of 95 Caucasian patients with hypospadias of different degrees (recruited at the Department of Pediatric Surgery) were selected for direct sequencing of the human *SF-1* gene in exons and flanking regions. Both strands were investigated using Big Dye terminator sequencing (v3.1, Applied Biosystems) and the ABI3730 Sequencer (Applied Biosystems). Chromatograms were analyzed by SeqScape v2.7 (Applied Biosystems). PCR was carried out in a volume of 25 μ l containing DNA (100–500 ng), MgSO_4 (1.5 mM), dNTP mixture (1.25 mM), Taq DNA polymerase (2.5 U), and 0.2 μ M of each primer. The PCR program used for amplification was as follows: 95°C for 2 min, (95°C for 45 s, 60°C for 30 s, 68°C for 2 min) 35 cycles. Primers were designed using the web-based Primer 3 program. The primer sequences are presented in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000343782). Cycle sequencing extension products were created in a final volume of 20 μ l reaction using 12.5 μ l H_2O , 0.5 μ l forward or reverse primer at 10 μ g/ml, 0.5 μ l template DNA, 4 μ l Big Dye Terminator Ready Reaction mix V1.1 (PE-ABI) and 2 μ l 5 $\times$ Big Dye buffer. Cycle sequencing conditions consisted of an initial denaturation step at 96°C for 1 min followed by 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Unincorporated dye and other contaminants were removed with ethanol precipitation procedure.

TaqMan and Statistical Analysis

We next investigated a total of 332 Caucasian hypospadias cases consisting of 78 penoscrotal, scrotal and perineal cases, which we here defined as severe, and 254 glandular, coronal, mid and distal penile cases that belong to the mild group, using the TaqMan analysis. The control group consisted of 422 healthy voluntary male blood donors from the Karolinska University Hospital. We investigated the missense p.G146A (c.437G>C) and the silent p.P125P (c.375C>T) polymorphism, respectively, located in the *SF-1* gene. Thus, polymorphism typing was performed using 5'-nuclease allele discrimination TaqMan assay together with fluorescent probes according to a standard protocol (Applied Biosystems). The results were analyzed on an ABI 7900HT. All statistical tests described below were performed by the PLINK program, version 1.07. Standard case/control allelic association test was performed, with missing data estimation. Odds ratio and its 95% confidence interval were also calculated. Furthermore, the p value was single-step adjusted using the Bonferroni method. Values of $p < 0.05$ (after adjustment with Bonferroni) were considered significant. Linkage disequilibrium of the 2 polymorphisms was further calculated. The Hardy-Weinberg test was also performed for each polymorphism. In addition, standard case/control genotypic association test was performed using Fisher's exact test.

Table 1. Genotypic distribution of the missense p.G146A (c.437G>C) and the silent p.P125P (c.375C>T) polymorphism in cases with mild and severe hypospadias, respectively, and in male controls

	Genotype frequency of p.G146A, n							Genotype frequency of p.P125P, n						
	CC		CG		GG		p value (unadjusted)	TT		TC		CC		p value (unadjusted)
	cases	controls	cases	controls	cases	controls		cases	controls	cases	controls	cases	controls	
Mild form	2	0	11	12	237	402	0.1212	0	0	1	1	251	416	1
Severe form	0	0	3	12	75	401	0.717	0	0	1	1	77	416	0.2906

Since hypospadias is considered to be a heterogeneous disease, the Caucasian cases were divided into 2 different groups, severe and mild, and accordingly analyzed.

Genomic DNA was extracted from either tissue harvested at surgery or from peripheral blood according to standard procedures reported elsewhere [Sambrook et al., 1989]. This study was approved by the ethics committee at Karolinska University Hospital. Informed written consent was obtained from all participants involved in the study.

Results

Direct Sequencing Analysis

Our initial direct sequencing analysis of the *SF-1* gene in the germline DNA from the male monozygotic Caucasian twins exhibiting very severe hypospadias, revealed a novel heterozygous missense mutation in c.938G>A, p.Arg313His. This mutation is located in the LBD. Upon further sequencing analysis of the DNA from the parents, the heterozygous mutation was also detected in the healthy father. To further explore the role of *SF-1* in the disease of hypospadias, this time in a sample set consisting of patients with varying degrees of hypospadias (i.e. mild and severe cases), the entire gene was screened in 95 cases. The direct sequencing analysis of the extended sample set revealed that there were genomic variations in 2 known SNPs. Thus, we found a silent polymorphism, p.P125P (c.375G>A) and a missense polymorphism, p.G146A (c.437G>C), respectively, in the hinge region of the *SF-1* gene. Interestingly, 1 case with severe hypospadias was a double heterozygote, and 1 mild case was homozygous for the p.G146A and heterozygous for the p.P125P polymorphism, respectively.

TaqMan Assay and Statistical Analysis, Respectively

Since several cases in the extended sample set (n = 95) displayed polymorphisms of the 2 known SNPs, we decided to further investigate the role of the p.P125P and p.G146A polymorphisms, respectively, in an even larger

patient sample set. Thus, a total of 332 patients with mild and severe degrees of hypospadias were screened for the 2 polymorphisms using TaqMan assays. This experiment included the 95 patients screened with the direct sequencing methodology. In addition, 422 male controls were included in the analysis. We found the TaqMan experiment to fully conform to the sequencing analysis in the 95 cases included in both experiments, and the success rate for genotyping the markers was 98%.

The TaqMan analysis revealed additional cases and controls, displaying a heterozygous or homozygous form of the 2 investigated SNPs in our large sample set, see table 1. No additional double heterozygotes were detected. Furthermore, the case/control genotypic association test (using Fisher's exact test) revealed no significance for each individual polymorphism investigated in the mild and the severe hypospadias group, respectively (mild group: c.437G>C (p.G146A), p = 0.1212; c.375C>T (p.P125P), p = 1; severe group: c.437G>C (p.G146A), p = 0.717; c.375C>T (p.P125P), p = 0.2906, table 1; both SNPs passed the Hardy-Weinberg test in the control group).

Moreover, the allelic association analysis of the 2 polymorphisms using PLINK did not show any significance in neither the mild nor severe hypospadias group (p values after Bonferroni adjustment were all above 0.05), see table 2. The p.G146A and p.P125P polymorphisms, respectively, locate 62 bp away from each other, and were not in linkage disequilibrium (R-sq = 0.139).

Discussion

In the present study, we detected a novel heterozygous p.Arg313His (c.938G>A) missense mutation located in the LBD of *SF-1* in monozygotic male twins displaying very severe hypospadias. We further found no association between the p.G146A and p.P125P polymorphisms of the *SF-1* gene and the disease of hypospadias in Caucasians.

Table 2. Allelic prevalence and comparison of the *SF-1* gene c.437G>C (p.G146A) and the c.375C>T (p.P125P) polymorphism, respectively, in mild (n = 254) and severe (n = 78) hypospadias cases versus male controls (n = 422)

CHR	Polymorphism	bp	A1	F_A	F_U	A2	CHISQ	P	OR	SE	L95	U95	BONF
Mild cases versus controls													
9	c.437G>C	127262802	C	0.03	0.01453	G	3.738	0.05319	2.098	0.3915	0.9739	4.519	0.1064
9	c.375C>T	127262864	T	0.001984	0.001199	C	0.1297	0.7187	1.656	1.415	0.1034	26.53	1
Severe cases versus controls													
9	c.437G>C	127262802	C	0.01923	0.01453	G	0.1929	0.6605	1.33	0.6515	0.371	4.769	0.3667
9	c.375C>T	127262864	T	0.00641	0.001199	C	1.77	0.1834	5.374	1.417	0.3344	86.38	1

A1 = Minor allele; A2 = major allele; CHR = chromosome; BONF = p value adjusted after the Bonferroni method; bp = base pair; CHISQ = χ^2 test; F_A = frequency for the minor allele in cases; F_U = frequency for the minor allele in controls; L95 = lower 95% confidence interval (CI) limit; OR = odds ratio; P = p value; Polymorphism = single nucleotide polymorphism; SE = standard error; U95 = upper 95% CI limit.

Table 3. Summary of results on the p.G146A polymorphism in different disorders and controls

	Adrenal disorders		Severe micropenis		Type 2 diabetes		Cryptorchism		Hypospadias		
	cases (n = 30)	control 1 (n = 55)	cases (n = 30)	control 2 (n = 115)	cases (n = 151)	control 3 (n = 141)	cases (n = 72)	control 4 (n = 136)	mild cases (n = 254)	severe cases (n = 78)	control 5 (n = 422)
C/C	4	2	2	8	24	11	4	8	2	0	0
G/C	10	5	15	24	64	55	34	35	11	3	12
C, %	30	8.2	31.7	17.4	37.1	27.3	29.2	18.8	3.0	1.9	1.4
Reference	Japanese individuals investigated by WuQiang et al. [2003]		Japanese individuals investigated by Wada et al. [2005]		Chinese Han individuals investigated by Liu et al. [2006]		Japanese individuals investigated by Wada et al. [2006]		Caucasians investigated in our present study		

Bold numbers indicate the percentage of controls exhibiting the G146A polymorphism.

Recent studies have reported heterozygous disruptive changes of the *SF-1* gene in a range of 46,XY disorder of sex development phenotypes, such as severe undermasculinization, mild or partial gonadal dysgenesis, impaired androgen synthesis, although, with apparently normal adrenal function [Correa et al., 2004; Hasegawa et al., 2004; Mallet et al., 2004; Coutant et al., 2007; Lin et al., 2007; Reuter et al., 2007; Köhler et al., 2008]. *SF-1* mutations have also been described in 46,XY adolescent females with primary amenorrhea and with low testosterone levels [Philibert et al., 2010]. Furthermore, Köhler et al. [2009] reported *SF-1* mutations in 3 cases with severe hypospadias, but not in the milder form of hypospadias. To date, a total of 45 *SF-1* mutations are reported in the Human Gene Mutation Database (<http://www.hgmd.org/>), and include missense/nonsense, splicing, small deletions/insertions/indels, gross deletions and complex rearrangements. In the current report, we present the iden-

tification of a novel missense mutation (p.Arg313His) located in the LBD of *SF-1* in 2 monozygotic male twins with very severe hypospadias. The mutation was also detected in the healthy father, although, since this specific mutation has previously not been reported, we hypothesized it may play a role in the development of hypospadias. In addition, quite recently, another study reported the identification of a novel p.Arg313Cys mutation in a patient with isolated distal hypospadias [Allali et al., 2011]. Moreover, several mutations in the LBD of *SF-1* have been reported in other studies, including cases with severe hypospadias [Ferraz-de-Souza et al., 2011]. Thus, we sequenced the *SF-1* gene in an extended Caucasian sample set, but found no further mutations. To clarify the role of the novel missense mutation (p.Arg313His) in the disease of hypospadias, additional studies are required.

The detected p.G146A polymorphism found in our extended sample set is located in the hinge region of *SF-1*

and has previously been described to mildly diminish the transactivation activity of SF-1 [WuQiang et al., 2003]. The polymorphism has further been proposed to act as a susceptibility factor for adrenal disease, cryptorchidism, severe micropenis, and type 2 diabetes [WuQiang et al., 2003; Wada et al., 2005, 2006; Liu et al., 2006]. To provide an overview of the previously published data regarding the p.G146A polymorphism, we summarize these findings in table 3. Comparing previously published results with our data, we notice a rather high difference regarding how common the C allele is in the investigated control populations. According to our data, the C allele has a prevalence of 1.4% in the control population, whereas the frequency of the same allele is approximately 5–18 fold increased in other control populations. This may be explained by ethnic group differences or simply by the insufficient number of individuals investigated in each study. Nevertheless, in all the published studies referred to in table 3, the C allele was found to be associated with

an increased risk of the investigated diseases. In our present study, we show that the C allele is not associated with the disease of hypospadias in Caucasians.

In conclusion, we have investigated the largest Caucasian cohorts of control and hypospadias cases with regard to the *SF-1* gene. Based on our data, we show that the p.G146A and p.P125P polymorphisms of the *SF-1* gene do not play an important role in the development of hypospadias with varying degrees in the investigated Caucasian population. To clarify the role of the novel missense mutation (p.Arg313His) in the disease of hypospadias, additional studies are required.

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