Genetic polymorphisms of 17β-hydroxysteroid dehydrogenase 3 and the risk of hypospadias

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ABSTRACT----------

Introduction. Hypospadias is a common congenital anomaly caused by incomplete fusion of urethral folds. Development of the urethra and external genital system in the male fetus is an androgen-dependent process. In this regard, enzymes 17β-hydroxysteroid dehydrogenase type 3 (17βHSD3, encoded by *HSD17B3*) and steroid 5α-reductase type 2 (encoded by *SRD5A2*) play crucial roles.

Aim. To investigate the possible associations between common polymorphisms in *HSD17B3* as well as well-known V89L polymorphism in *SRD5A2* and risk of hypospadias.

Methods. A case-control study was performed between 1999 and 2005. There were 89 Japanese boys with hypospadias and 291 newborn controls. We genotyped *HSD17B3* –1999T>C, +10A>G, +20A>G, +139G>A (V31I), +913G>A (G289S) and *SRD5A2* +336G>C (V89L) polymorphisms by allelic discrimination assay. We measured mRNA expression of the wildtype G289 allele and the mutant S289 allele of the *HSD17B3* gene in the transfected human fetal kidney HEK293 cells.

Main Outcome Measures. Assessment of Hypospadias including its severity and *HSD17B3* and *SRD5A2* genes using DNA blood samples: allele and genotype distribution of SNPs in these two genes in cases and controls.

Results. In our study, the risk of hypospadias was significantly higher in subjects carrying homozygous *HSD17B3* +913A (289S) alleles (odds ratio (OR): 3.06; 95% confidence intervals (CI): 1.38-6.76). The risk of severe hypospadias was much higher in these subjects (OR: 3.93; 95% CI: 1.34-11.49). The mRNA expression levels of *HSD17B3* G289 was higher than that of *HSD17B3* S289 mutant ($P < 0.001$). In addition, the risk of severe hypospadias increased in boys carrying the *SRD5A2* +336C (89L) allele (OR: 3.19; 95% CI: 1.09-9.36).

Conclusions. These results suggest that the *HSD17B3* G289S polymorphism may be a potential risk modifier for hypospadias. Our findings provide evidence that a certain genotype related to androgen production may potentiate risk of hypospadias.

Key words. Hypospadias; androgens; polymorphism; 17β-hydroxysteroid dehydrogenase; development of the penis

Introduction

The development of the male external genitalia is a complex process, comprising genetic programming, cell differentiation, hormonal signaling including paracrine and autocrine regulation, enzyme activity, and tissue remodeling, which follows an orderly sequence, occurring in a time- and concentration dependent way [1,2]. Moreover, balance between androgens and estrogens seems to be important to the proper male genital development [1]. For example, activating transcription factor 3 (ATF3), an estrogen responsive gene, has been reported to be expressed during sexual development and up-regulated in hypospadic genital skin, whereas the oxytocin receptor (OTR) gene has been reported to be highly expressed and might be involved in coordinating timely and appropriate proliferation and migration of the penile cells [1,3]. Any disturbance in these processes and/or in balance between androgens and estrogens may lead to hypospadias.

Hypospadias is characterized by incomplete fusion of the urethral folds and is associated with various degrees of chordee deformity [4]. The prevalence of hypospadias varies widely between different countries and populations, ranging from 0.26 to 47 per 10,000 infants [5,6]. The prevalence in Hokkaido, Japan, is approximately 3.9 per 10,000 infants [7]. Hypospadias occurs as a multifactorial disorder subject to various genetic and environmental factors [8,9]. As development of the urethra and external genital system in the male fetus is an androgen-dependent process, abnormalities in the synthesis and metabolism of androgens can result in abnormal genital development.

The enzymes 17β-hydroxysteroid dehydrogenase type 3 (17βHSD3, encoded by *HSD17B3*) and steroid 5α-reductase type 2 (encoded by *SRD5A2*) are involved in the synthesis and metabolism of androgens [10]. *HSD17B3* is located on chromosome 9q22 and comprises 11 exons [10,11]. In the testis, the 17βHSD3 isozyme catalyzes the conversion of the C19-steroid, androstenedione, to the biologically more active androgen, testosterone [10]. A missense substitution of glycine (G) to serine (S) at codon 289 (G289S) in *HSD17B3* results from a single nucleotide polymorphism (SNP) in exon 11 [12]. The G289S polymorphism seems apparently silent (non-deficient) [12], and may be associated with susceptibility to prostate cancer, in which it is well established that androgens play an important role [13]. Steroid 5α-reductase type 2 is encoded by *SRD5A2* on chromosome 2p23 [14]. This enzyme is

involved in male sex differentiation by converting testosterone to 5α-dihydrotestosterone (DHT) in peripheral target tissues. It is believed that polymorphic variants of *SRD5A2* influence 5α-reductase type 2 activity. In particular, a polymorphism of *SRD5A2*, consisting of a substitution of valine (V) to leucine (L) at codon 89 (V89L) in exon 1, is associated with different levels of steroid 5α-reductase type 2 activity [15]. This polymorphism is associated with a partial reduction in enzymatic activity. Both 17βHSD3 and steroid 5α-reductase type 2 deficiencies cause 46,XY disorders of sex development, in which the external genitalia are undervirilized [10-12,16-24]. Very rare mutations in *HSD17B3* or *SRD5A2* are responsible for 46,XY disorders of sex development; from various ethnic groups more than 20 different mutations in *HSD17B3* and more than 40 different mutations in *SRD5A2* have been reported [10,11,19,21,25,26].

Previous studies have shown that mutations in *SRD5A2* result in genital malformations such as hypospadias [27,28], and have suggested that the androgen milieu is abnormal in boys with hypospadias [29,30]. Recently, it was reported that the common V89L polymorphism in *SRD5A2* is associated with hypospadias [31,32], and that suggestive linkage was found at the chromosome region 9q22 in which *HSD17B3* is located [33]. Thus, hypospadias may be caused by mutations in *HSD17B3* or *SRD5A2*, which would result in insufficient androgen production/action in the male fetus. In this study, by genotyping common five *HSD17B3* polymorphisms as well as well-known *SRD5A2* polymorphism previously reported, in 89 Japanese boys with hypospadias and 291 Japanese controls and examining mRNA expression of the most susceptible polymorphism, we tested the hypothesis that *HSD17B3* polymorphisms may be associated with hypospadias.

Materials and Methods

Subjects

This case-control study was performed in the Hokkaido, co-operating with a few hospitals in Japan between 1999 and 2005 [34,35]. 94 cases were defined as males who underwent surgery for hypospadias at the Department of Urology of Hokkaido University Hospital, Saitama Children's Medical Center, Chukyo Hospital or Tokushima University Hospital. Information about the severity of hypospadias in the patient group was obtained from hospital records. Five patients were excluded from this study because one patient had a brother with hypospadias and four patients had syndromes (Klippel-Trenaunay-Weber syndrome (n = 1), VATER syndrome (n = 1), mixed gonadal dysgenesis (n = 2)). Hypospadias is classified as mild (distal) when the opening of the urethra is in the penile, coronal or glanular portion, and as severe (proximal) when the opening of the urethra is in the penoscrotal, scrotal or perineal portion. The urologists who operated on the patients classified 89 cases by degree of hypospadias into 56 mild cases and 33 severe ones. Control group was composed of 291 male newborn infants without any malformation who had been born at the obstetrics departments of hospitals in the Hokkaido [35]. Obstetricians in these hospitals carefully examined whether newborns had any congenital malformations according to the manual of monitoring for major markers of congenital malformations including hypospadias. All participants were native Japanese. The study was conducted with the informed consent of the parents of subjects and was approved by the Institutional Ethical Board for Human Gene and Genome Studies of Hokkaido University Graduate School of Medicine.

Selection and determination of HSD17B3 and SDR5A2 polymorphisms

As shown in Table 1, we selected five common polymorphisms of *HSD17B3* whose minor allele frequency was at least 5.0% in a Japanese population according to the available SNP databases such as dbSNPs and the International Hapmap Project [36], as well as a well-known *SRD5A2* polymorphism that might be susceptible to risk of hypospadias [31,32]. These were *HSD17B3* –1999T/C, +10A/G, and +20A/G polymorphisms at 1999 bp upstream, 10 and 20 bp downstream from the transcription start site, respectively; +139G/A (V31I) and +913G/A (G289S) polymorphisms at 139 and 913 bp downstream from the transcription start site in the mRNA, respectively; and the *SRD5A2* +336G/C

(V89L) polymorphism at 336 bp downstream from the transcription start site in the mRNA (Table 1). Each polymorphism could result in a functional change based on location in either the coding region, with an amino acid substitution, or in the 5′-flanking region, especially in the transcription factor binding site or in the exonic splicing enhancers.

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp or EZ1 DNA Blood kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Genotyping of each polymorphism was performed by allelic discrimination using fluorogenic probes and the 5'nuclease (TaqMan) assay as described [37]. TaqMan SNP Genotyping Assays for each polymorphism were obtained from Applied Biosystems (Foster City, CA). All probe-primer sets were designed to function using universal reaction and cycling conditions. Genotyping was performed in 10 µl reactions containing approximately 40 ng genomic DNA, 0.5 µl 20× TaqMan SNP Genotyping Assay Mix (consisting of unlabeled PCR primers, and 6-carboxy-fluorescein (FAM) and vasoactive intestinal contractor (VIC) dye-labeled TaqMan minor groove binder (MGB) probes), and 5.0 µl of 2× Taqman Universal PCR Master Mixture. Wells from these reactions were read using an Applied Biosystems 7500 Real-Time PCR System at 490/520 nm excitation/emission (FAM) and 530/560 nm excitation/emission (VIC). Cycling conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 92°C for 15 s and 60°C for 1 min. FAM and VIC fluorescence levels of PCR products were measured at 60ºC for 1 min. Samples were genotyped using allelic discrimination in an Applied Biosystems 7500 Real-Time PCR System.

Functional analysis of HSD17B3 G289S polymorphism

We examined whether there might be any differences in mRNA expression between the wildtype G289 allele and the mutant S289 allele of HSD17B3 by the following method of transfection. The expression plasmid pCMV-*HSD17B3* (pCMV-*HSD17B3*_G289) was purchased from OriGene (Rockville, MD, USA). The G289S mutant of *HSD17B3* (pCMV-*HSD17B3*_ S289) was generated in plasmid pCMV using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) with mutagenic primers *HSD17B3*_S289_P1: 5′-GGG CCT TCT ACA GCA GTG CCT TCC AAA GGC-3′ and *HSD17B3*_

S289_P2: 5′-GCC TTT GGA AGG CAC TGC TGT AGA AGG CCC-3′. The G289S mutation was verified by DNA sequencing of the entire clone.

Human fetal kidney HEK293 cells were cultured in 6 well plates at 37ºC in an atmosphere of 5% CO_2 and 100% humidity. For maintenance of the cells, DMEM (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% bovine serum albumin (Moregate, Australia), penicillin, streptomycin and amphotericin B (antibiotic antimycotic; GIBCO BRL) was used.

HEK293 cells were transfected simultaneously with 1ug of pCMV-*HSD17B3*_G289 or pCMV-*HSD17B3*_S289 plasmid. The transfection was performed using FuGene 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction manual. The cells were collected from 3 wells at 24, 25 and 29 hrs after the transfection, respectively. Total RNA was extracted from cells using TRI reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instruction manual. RNA (0.1 µg) was reverse-transcribed to cDNA using the RT enzyme. *HSD17B3* and *GAPDH* mRNA were assayed using an ABI StepOnePlus (Applied Biosystems, USA). The specific primers and TaqMan probes of *HSD17B3* and *GAPDH* were purchased as pre-developed gene expression assays from Applied Biosystems. A 20 µl aliquot of the Universal PCR Master Mix (TOYOBO, Osaka, Japan) containing an appropriate amount of the template cDNA, primers, TaqMan probe was prepared, and real-time PCR was performed by 50 cycles of incubation at 95ºC for 15 sec, followed by incubation at 60ºC for 1 min. Data were analyzed using the comparative Ct method.

Statistical Analysis

We calculated odds ratios (ORs) and 95% confidence intervals (CIs) associated with *HSD17B3* and *SRD5A2* genotypes by unconditional logistic regression analysis. Furthermore, a gene dosage effect of the number of alleles was assessed by modeling a linear effect on the log odds scale for each allele in a logistic regression model. Additionally, multiple comparisons (Bonferroni correction) were carried out on these six of *HSD17B3* and *SRD5A2* genotypes as described [38,39]. Hardy-Weinberg equilibrium analyses were performed to compare observed and expected genotype frequencies using the chi-square test. The haplotype was analyzed using Haploview version 4.0 based on the expectation-maximization

algorithm [40], and linkage disequilibrium between loci was measured using Lewontin's D' [41]. Statistical analyses in the experiment were performed by Tukey-Kramer HSD. The values were expressed as the mean value ± standard error (SE), and the difference at a level of significance less than 0.05 ($P < 0.05$) was regarded as statistically significant as well as others. In multiple comparisons, Bonferroni threshold was 0.0083. Statistical analyses were conducted using SPSS software for Windows version 15.0 (SPSS, Chicago, IL, USA).

Results

We compared the genotypic frequencies of *HSD17B3* –1999T/C, +10A/G, +20A/G, +139G/A, +913G/A and *SRD5A2* +336G/C polymorphisms among 89 hypospadias cases and 291 newborn controls in a Japanese population (Table 2). The distribution of genotypes in each group was in Hardy-Weinberg equilibrium (chi-square test, $P > 0.05$). We found a significantly higher risk for hypospadias in the *HSD17B3* +913G/A polymorphism group. Thirty-seven boys (41.6%) with hypospadias had heterozygous A alleles compared with 108 controls (37.1%); thirteen boys (14.6%) with hypospadias had homozygous A alleles compared with 18 controls (6.2%). The risk of hypospadias was significantly higher in boys carrying the *HSD17B3* +913A (289S) alleles with a gene dosage effect (GG genotype [reference]; GA genotype, OR: 1.45, 95% CI: 0.87-2.42; AA genotype, OR: 3.06, 95% CI: 1.38-6.76 [*P* for trend: 0.007]; GA + AA genotypes, OR: 1.68, 95% CI: 1.04-3.71). On the other hand, we found no significant difference between the distributions of other genotypes in the two groups ($P > 0.05$).

We also investigated the association between the severity of hypospadias and these polymorphisms (Table 2). The risk of severe hypospadias was significantly higher in boys carrying the *HSD17B3* +913A alleles with a gene dosage effect (GG genotype [reference]; GA genotype, OR: 1.42, 95% CI: 0.64-3.12; AA genotype, OR: 3.93, 95% CI: 1.34-11.49 [*P* for trend: 0.026]). Similarly, the risk of mild hypospadias was modestly higher in boys carrying the *HSD17B3* +913A alleles (GG genotype [reference]; GA genotype, OR: 1.47, 95% CI: 0.80-2.70; AA genotype, OR: 2.57, 95% CI: 0.97-6.77 [*P* for trend: 0.047]) with borderline statistical significance. In addition, the risk of severe hypospadias was higher in boys carrying the *SRD5A2* +336C (89L) alleles (GG genotype [reference]; GC genotype, OR: 3.16, 95% CI: 1.05-9.50; CC genotype, OR: 3.29, 95% CI: 0.95-11.47 [*P* for trend: 0.057]; GC + CC genotypes, OR: 3.19, 95% CI: 1.09-9.36).

To correct the significance level according to multiple comparisons, we compared the observed *P* values with the Bonferroni threshold as described in Materials and methods. The observed *P* value for G289S in *HSD17B3* for hypospadias was lower than the Bonferroni threshold ($P = 0.0083$), whereas other genotypes including V89L in *SRD5A2* for severe hypospadias were beyond it.

There was only one haplotype block identified in the present study; *HSD17B3* +10A/G and

+20A/G located on chromosome 9q22 (Table 3). We observed the highest degree of linkage disequilibrium between polymorphisms *HSD17B3* +10A/G and +20A/G (D' = 0.97). We found no significant differences in any haplotype frequencies of the block (Table 3).

At 29 hours after the transfection, we found the significant difference in the expression levels of wild and substituted types of *HSD17B3* mRNA (Figure 1). The mRNA expression levels of *HSD17B3* G289 was higher than that of *HSD17B3* S289 mutant ($p<0.001$).

Discussion

To the best of our knowledge, this is the first report to investigate the *HSD17B3* locus as a candidate gene for hypospadias susceptibility. The 17βHSD3 enzyme is selectively expressed in the testis and catalyzes the reduction of androstenedione to the biologically more active androgen, testosterone. Testosterone acts via the androgen receptor in the Wolffian duct to initiate development of the male internal genitalia (epididymis, vas deferens, seminal vesicles, and ejaculatory ducts). A 17βHSD3 deficiency causes 46,XY disorders of sex development [10-12,17-21,24], in which the external genitalia is undervirilized (often a female phenotype with or without clitoromegaly and/or labial fusion and a blind-ending vagina). It was reported that the clinical picture of 17βHSD3 deficiency may be characterized by overt ambiguous external genitalia, male genitalia with micropenis, or isolated hypospadias; in these infants, male sex is assigned and they are reared accordingly [10,11,19,42-47]. As the management of these disorders contained various issues including biological, ethical and social ones, psychosexual development in these disorders as well as hypospadias has been reported [48-50]. This phenotype is caused by inadequate testicular testosterone synthesis in the anlage of the external genitalia during fetal development. Recently, genome-wide linkage analysis revealed that chromosome 9q22, on which *HSD17B3* is located, might be one of the susceptible regions for hypospadias [33]. However, it was reported that there were only two cases with heterozygosity for G289S polymorphism among 158 European cases with hypospadias [32]. The frequency of *HSD17B3* G289S polymorphism in the European is remarkably lower than that in the Japanese. According to the HapMap project, only 4.2% of the European had the A (S289) allele, whereas 22.7% of the Japanese had it. Thus, *HSD17B3* polymorphisms vary with ethnicity. Our result derived from 380 individuals is regarded as a representative sample of the Japanese population, because the Japanese people are believed to be genetically homogeneous except for minor indigenous populations that are estimated at most 0.02% of the whole population [51]. Additionally, if another control group composed of 101 male healthy volunteer students aged between 18 and 24 years is adopted, the risk of hypospadias in subjects carrying homozygous HSD17B3 +913A (S289) alleles will be similar (OR: 3.28; 95% CI: 1.15-9.35). We investigated the association in a Japanese population between five common *HSD17B3* polymorphisms

and hypospadias. The G289S mutation was previously reported in heterozygous form in normal women and in women with polycystic ovary syndrome [12]. In vitro studies, demonstrated that enzymes bearing either glycine or serine at this position have similar substrate specificities and kinetic constants [12]. However, Margiotti *et al.* found that G289S polymorphism confers a significant increase in prostate cancer risk, reported two men with prostate cancer homozygous for this mutation, and suggested that such a variant may be associated with increased androgenic activity [13]. On the other hand, it was reported that an Italian patient whose prenatal diagnosis of a 46,XY foetus with female external genitalia had homozygous G289S mutations, but impaired plasma testosterone level [52]. Thus, S289 may be associated with impaired androgenic activity, which consistent with our in vitro study. We also found a gene dosage effect between the number of susceptible S289 alleles and the risk of hypospadias (*P* for trend = 0.007). Such a trend in the *HSD17B3* G289S polymorphism was found in more severe form of hypospadias. However, there was a limitation in the study. The number of patients of this case-control study was limited. Consequently, our sample size was slightly lack of power. According to the frequencies observed in this study, to detect statistical significance with 80% power (α = 0.05) using a chi-square test, about 147 cases with 294 controls or 207 cases with 207 controls would be needed for the *HSD17B3* G289S polymorphism.

The enzyme steroid 5α-reductase type 2 is involved in male sex differentiation by converting testosterone to DHT. Previous studies have shown that *SRD5A2* mutations result in genital malformations such as hypospadias, especially of the severe type [27]. Also, in utero administration of the steroid 5α-reductase inhibitor finasteride induces hypospadias and other sexual differentiation abnormalities in rats and monkeys [53-55]. Additionally, the connection between 5α-reductase inhibitors and sexual adverse effects in humans has been established in the literature [56]. Silver and Russell suggested that a partial deficiency of steroid 5α-reductase type 2 activity may explain isolated hypospadias [28]. Wang *et al.* suggested that the presence of leucine (C allele) of V89L in *SRD5A2* is a genetic risk factor for hypospadias [31]. Similarly, Thai *et al.* reported that the valine (G allele) of the V89L polymorphism in *SRD5A2* confers a reduced risk for hypospadias [32]. Moreover, it has been well established that genetic polymorphisms determine functional increases or decreases in catalytic

activity of steroid 5α-reductase type 2 or drug response to specific inhibitors and especially the V89L polymorphism in *SRD5A2* has been functionally well characterized as producing a physiologic low enzymatic activity and that it is a relatively common genetic variant [15,57,58]. In the present study, a similar association was found only for severe hypospadias. These results are supported by the finding that the V89L substitution in steroid 5α-reductase type 2 causes an approximate 30% reduction in enzyme activity [15]. Actually, it was reported that the genotype of VL showed 85% activity of that of VV wild type, and LL genotype showed 72% activity of wild type [15]. In other experiment, V89L variant has reported to show moderately reduced enzyme activity, suggesting that the low activity of V89L is mainly determined by the LL homozygote genotype, which is compatible with a recessive hereditary model of transmission [57]. Kim *et al.* reported that steroid 5α-reductase type 2 mRNA localizes to the stroma of the corpora cavernosa, spongiosum, skin, and inner prepuce in the human male fetus between 16 and 20 weeks of gestation, and that the localization of this enzyme in the central stroma of the remodeled urethral seam is thought to play an important role in the final fusion process of the urethral folds into the tubular urethra [59]. Moreover, the LL genotype of the V89L polymorphism was associated with significantly lower concentrations of testosterone and free testosterone (by 12% and 16%, respectively) and an 8% higher sex hormone-binding globulin concentration in British men [60].

Given that DHT is 50-fold more potent than testosterone [16], it is plausible that homozygosity for the *SRD5A2* 89L (C allele)—involved in testosterone conversion to DHT—increases the risk of severe hypospadias. Nonomura *et al.* reported that the testosterone response to human chorionic gonadotropin stimulation in hypospadias patients was lower than that in normal controls and that the basal luteinizing hormone levels in proximal (severe) hypospadias were higher than those in distal (mild) hypospadias, suggesting a direct correlation between severe hypogonadism and severe hypospadias [30]. Raboch *et al.* studied peri-pubertal boys and young men with severe hypospadias and found reduced average serum testosterone levels [61], whereas Gearhart *et al*. found that adult patients with mild hypospadias had normal testosterone levels but elevated luteinizing hormone levels [62]. Thus, they suggested that the severity of hypospadias may depend on the action of testosterone. We

could detect significant differences in the *SRD5A2* V89L polymorphism between the severe type of cases and controls (Table 2). Unfortunately, our results became non-significant when multiple testing is taken into consideration (Bonferroni correction). However, this correction is a controversial matter because type II errors (the probability of accepting a null hypothesis, when it is actually false) tend to increase in an attempt to cut down type I errors (the probability of rejecting the null hypothesis, when it is indeed true) by applying the Bonferroni correction. This means that significant results are lost and the power of the study is reduced. [63]. Anyhow, further studies with a larger population are needed to elucidate the association between polymorphisms in *SRD5A2* and hypospadias.

Conclusions

Our results suggest an association between genetic polymorphisms in *HSD17B* and the risk of hypospadias. This finding provides evidence that genetic factors related to testosterone biosynthesizing enzymes may affect the risk of hypospadias. This is the first report of an association between hypospadias and the *HSD17B3* G289S polymorphism. Therefore, the role of androgen-related polymorphisms should be investigated in biochemical and kinetic detail to determine their precise functional significance in a larger-scale population.

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LEGEND

Figure 1 mRNA levels of *HSD17B3* after transfection of *HSD17B3* expression plasmid. ■ indicates the wildtype G289 allele and □ indicates the variant S289 allele. There is a significant difference in the expression levels of wild and variant types of *HSD17B3* mRNA at 29 hours after the transfection ($p<0.001$ Tukey-Kramer HSD).

Figure 1

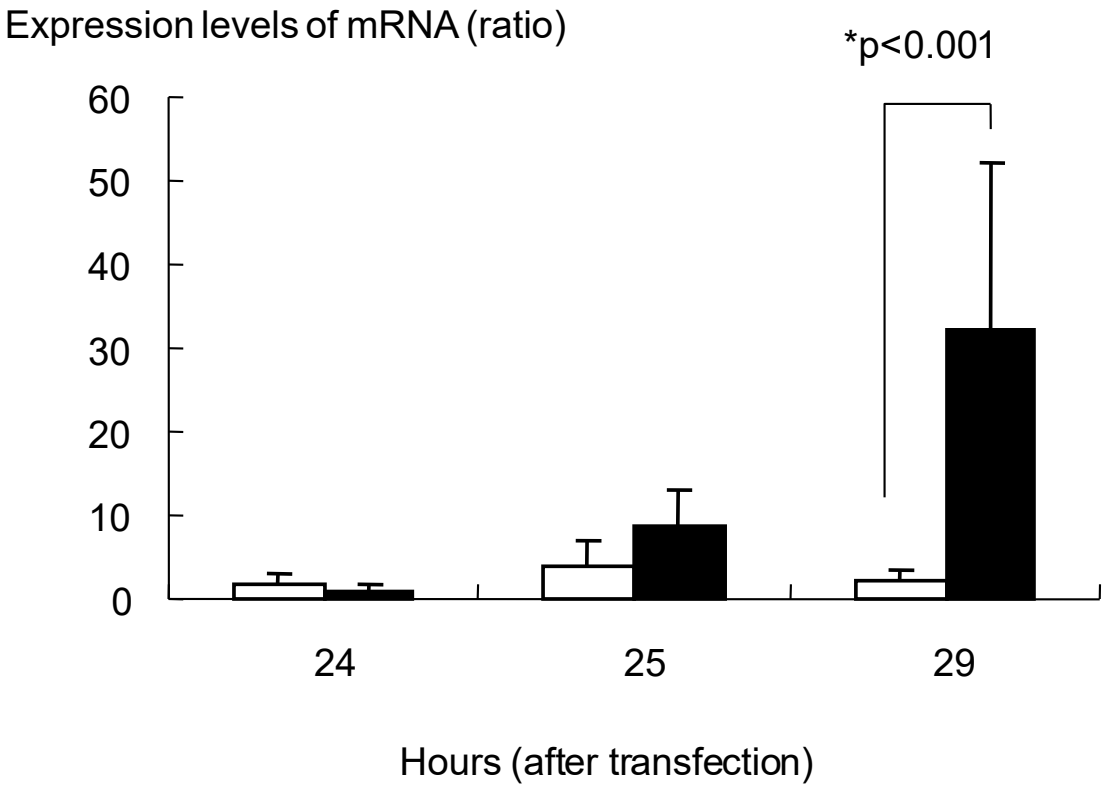
Expression levels of mRNA (ratio)
*p<0.001
60
50
40
30
20
10
0
24
25
29
Hours (after transfection)

Table 1 Five *HSD17B3* and one *SRD5A2* polymorphisms and allele frequencies in our control group

Gene	dbSNP ID	SNP position	SNP region	Minor allele frequency (%)
HSD17B3	rs4743709	–1999T>C	loc, tfbs	25.9
	rs2066474	+10A>G	utr, ese	41.9
	rs2066476	+20A>G	utr, ese	19.1
	rs2066480	+139G>A (V31I)	ns	4.1
	rs2066479	+913G>A (G289S)	ns, ese	24.7
SRD5A2	rs523349	+336G>C (V89L)	ns	44.0

loc: locus region; tfbs: transcription factor binding sites; utr: untranslated region; ese: exonic splicing enhancers; ns: coding-nonsynonymous (GeneCards: http://www.genecards.org/)

Table 2 Associations of five common *HSD17B3* and one well-known *SRD5A2* polymorphisms with hypospadias

Genotypes	Control (291)		Hypospadias (89)		OR	95% CI	*P* value	Mild Hypospadias (56)		OR	95% CI	*P* value	Severe Hypospadias (33)		OR	95% CI	*P* value
	No.	%	No.	%				No.	%				No.	%			
HSD17B3																	
–1999T/C																	
TT	156	53.6	48	53.9	1.00	reference		30	53.6	1.00	reference		18	54.5	1.00	reference	
TC	119	40.9	32	36.0	0.87	0.53-1.45	0.60	19	33.9	0.83	0.45-1.55	0.56	13	39.4	0.95	0.45-2.01	0.89
CC	16	5.5	9	10.1	1.83	0.76-4.40	0.18	7	12.5	2.28	0.86-6.00	0.097	2	6.1	1.10	0.23-5.10	0.92
						P for trend	0.57				*P* for trend	0.44				*P* for trend	0.97
+10A/G																	
AA	98	33.7	26	29.2	1.00	reference		16	28.6	1.00	reference		10	30.3	1.00	reference	
AG	142	48.8	40	44.9	1.06	0.61-1.85	0.83	25	44.6	1.08	0.55-2.13	0.83	15	45.5	1.04	0.45-2.40	0.94
GG	51	17.5	23	25.8	1.70	0.88-3.27	0.11	15	26.8	1.80	0.83-3.94	0.14	8	24.2	1.54	0.57-4.14	0.39
						P for trend	0.14				*P* for trend	0.16				*P* for trend	0.44
+20A/G																	
AA	192	66.0	64	71.9	1.00	reference		41	73.2	1.00	reference		23	69.7	1.00	reference	
AG	87	29.9	22	24.7	0.76	0.44-1.31	0.32	14	25.0	0.75	0.39-1.45	0.40	8	24.2	0.77	0.33-1.78	0.54
GG	12	4.1	3	3.4	0.75	0.21-2.74	0.66	1	1.8	0.39	0.05-3.09	0.37	2	6.1	1.39	0.29-6.61	0.68
						P for trend	0.32				*P* for trend	0.24				*P* for trend	0.86
+139G/A (V31I)																	
GG	269	92.4	83	93.3	1.00	reference		52	92.9	1.00	reference		31	93.9	1.00	reference	
GA	20	6.9	6	6.7	0.97	0.38-2.50	0.95	4	7.1	1.04	0.34-3.15	0.95	2	6.1	0.87	0.19-3.89	0.85
AA	2	0.7	0	0.0	0.00	-	1.00	0	0.0	0.00	-	1.00	0	0.0	0.00	-	1.00
						P for trend	0.67				*P* for trend	0.80				*P* for trend	0.69
+913G/A (G289S)																	
GG	165	56.7	39	43.8	1.00	reference		25	44.6	1.00	reference		14	42.4	1.00	reference	
GA	108	37.1	37	41.6	1.45	0.87-2.42	0.16	24	42.9	1.47	0.80-2.70	0.22	13	39.4	1.42	0.64-3.14	0.39
AA	18	6.2	13	14.6	**3.06**	**1.38-6.76**	**0.006**	7	12.5	2.57	0.97-6.77	0.057	6	18.2	**3.93**	**1.34-11.49**	**0.012**
						***P* for trend**	**0.007**				***P* for trend**	**0.047**				***P* for trend**	**0.026**
SRD5A2																	
+336G/C (V89L)																	
GG	89	30.6	23	25.8	1.00	reference		19	33.9	1.00	reference		4	12.1	1.00	reference	
GC	148	50.9	49	55.1	1.28	0.73-2.25	0.39	28	50.0	0.89	0.47-1.68	0.71	21	63.6	**3.16**	**1.05-9.50**	**0.041**
CC	54	18.6	17	19.1	1.22	0.60-2.48	0.59	9	16.1	0.78	0.33-1.85	0.57	8	24.2	3.29	0.95-11.47	0.061
						P for trend	0.53				*P* for trend	0.56				*P* for trend	0.057

OR odds ratio; CI confidence interval
Mild Hypospadias: the opening of the urethra is in the penile coronal or glanular portion
Severe Hypospadias: the opening of the urethra is in the penoscrotal scrotal or perineal portion

Table 3 Association of groups of *HSD17B3* haplotypes with hypospadias

+10A/G, +20A/G	Haplotype Frequency Estimation				
	Over all (760 alleles) (%)	Hypospadias (178 alleles) (%)	Controls (582 alleles) (%)	Chi-square	*P* value
GA	43.3	48.3	41.9	2.28	0.13
AA	38.6	37.1	39.1	0.22	0.64
AG	17.8	14.0	19.0	2.34	0.13
GG	0.2	0.6	0.1	2.03	0.15