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Review Article

Molecular Epidemiology of Hypospadias: Review of Genetic and Environmental Risk Factors

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Hypospadias is one of the most common congenital anomalies in the United States, occurring in approximately 1 in 125 live male births. It is characterized by altered development of the urethra, foreskin, and ventral surface of the penis. In this review, the embryology, epidemiology, risk factors, genetic predisposition, and likely candidate genes for hypospadias are described. Recent reports have identified increases in the birth prevalence of mild and severe forms of hypospadias in the United States from the 1960s to the present. Studies in consanguineous families and small case series have identified allelic variants in genes controlling androgen action and metabolism that cause hypospadias, but the relevance of these findings to the general population is unknown. Concern has also focused on whether exposure to endocrine disrupting chemicals (EDC) with antiandrogenic activity is the cause of this increase. Hypospadias is believed to have a multifactorial etiology in which allelic variants in genes controlling androgen action and metabolism predispose individuals to develop this condition. When genetic susceptibility is combined with exposure to antiandrogenic agents, a threshold is surpassed, resulting in the manifestation of this birth defect. A clear role for exposure to antiandrogenic environmental chemicals has yet to be established in the etiology of hypospadias, although results from laboratory animal models indicate that a number of environmental chemicals could be implicated. Molecular epidemiology studies that simultaneously examine the roles of allelic variants in genes controlling androgen action and metabolism, and environmental exposures are needed to elucidate the risk factors for these anomalies and the causes of the increased rate of hypospadias. *Birth Defects Research (Part A) 67:825–836, 2003.* © 2003 Wiley-Liss, Inc.

INTRODUCTION

Molecular Epidemiology of Birth Defects

Recent epidemiologic approaches have utilized molecular tools to identify interactions between genetic and environmental factors in the causation of complex diseases. Many single gene disorders are characterized by a low frequency of the disease allele in the general population and high penetrance (i.e., a large proportion of individuals with the disease allele develop the disorder). The susceptibility genes for these single gene disorders are usually rare in the general population (allele frequency <1%), typically demonstrate Mendelian patterns of inheritance, and are associated with high disease risk (Rothman et al., 1995). From over 2000 likely single gene birth defect syndromes in humans, the gene has been isolated in only 5% and mapped in a further 5% (Winter, 1996). By comparison, in the mouse there are approximately 500 spontaneously occurring single gene defects associated with birth defects; approximately 15% of these genes have been found, and over 80% have been mapped in the mouse. Despite the availability of mouse mutants and the identification of genes that are important in the development of worms, flies, frogs, and fish, the study of genes and human birth defects has not received comparable experimental attention. However, genes that have been identified as important for morphogenesis in these experimental species provide a rich source of information about potential susceptibility genes in humans.

Multifactorial disorders, in contrast, are characterized by high levels of genetic complexity and probable gene-by-environment interactions (Ellsworth et al., 1997). Although they are not usually inherited in a simple Mendelian fashion, multifactorial disorders tend to aggregate within families. Susceptibility genes for multifactorial disorders tend to be common in the general population (allele frequency ≥1%), and are usually associated with a low risk for complex disease. However, these susceptibility genes may interact with specific environmental factors to produce a markedly increased risk of disease. In this review we use the term "allelic variants" to describe both common and

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Figure 1. Example of ventral chordee associated with hypospadias.

Figure 3. Middle hypospadias on the penile shaft.

rare mutations in genes associated with the birth defect hypospadias. This condition is governed by a spectrum of genetic and environmental factors. There have been reports of an increased prevalence of hypospadias and other disorders of the male genital tract due to environmental exposure to xenoestrogens and/or antiandrogens (Sultan et al., 2001). The purpose of this review is to examine the literature on known genetic and environmental risk factors for hypospadias, and to make recommendations as to what types of studies are needed to identify the causes of the increased prevalence of hypospadias.

Embryology of Hypospadias

The term "hypospadias" refers to an opening on the ventral side of the penis, while "epispadias" is an opening on the dorsal side. Hypospadias denotes a condition in which the urethra has failed to completely form, and is often associated with a ventral curvature of the penis (chordee) (Fig. 1). Hypospadias is classified according to severity. The first degree is the mildest form, and the urethra opens on the anterior portion of the penis (glandular and subcoronal; see Fig. 2). The second degree is more severe and involves openings on the midshaft of the penis (Fig. 3). The third degree is the most severe and involves posterior penile, penoscrotal, scrotal, and perineal openings (Fig. 4). First-degree hypospadias accounts for approximately 50% of cases, second-degree for 30%, and third-degree for 20% (Fig. 5) (Levitt and Reda, 1988; Duckett and Baskin, 1996).

The development and elongation of the phallus occurs between weeks 8 and 14 of prenatal life under the influence of the androgens testosterone (T) and 5α-dihydrotestosterone (DHT), which are synthesized in response to a surge of luteinizing hormone (LH) from the fetal pituitary (Moore, 1988; Bingol and Wasserman, 1990). At the end of the third month, the urethral folds close over the urethral plate to form the penile urethra. The urethral canal does not extend to the glans, or the tip of the phallus, until the fourth month, when ectodermal cells derived from the tip of the glans penetrate inward and form a short epithelial cord (Van der Werff et al., 2000). The penile urethra forms as a result of the medial edges of the endodermal urethral folds fusing to form the median raphe (Baskin, 2000).

The diagnosis of hypospadias is apparent on examination of newborns. The incomplete formation of the prepuce with an excess dorsal hood leads to an immediate recognition of the defect. A diagnosis of hypospadias can be made by prenatal ultrasound, and typical findings are a wide distal end of the penis that correlates with the excess dorsal prepuce (Duckett and Baskin, 1996). The only treatment for hypospadias is surgical repair, and reconstruction generally involves a single outpatient procedure performed after 6 months of age. Considerable advances have been made in surgical repair procedures for hypospadias, and there is an excellent prognosis for good cosmetic and functional results in infants with all degrees of hypospadias (Duckett and Baskin, 1996).

Figure 2. Anterior hypospadias at the coronal margin.

Figure 4. Posterior hypospadias at the level of the perineum.

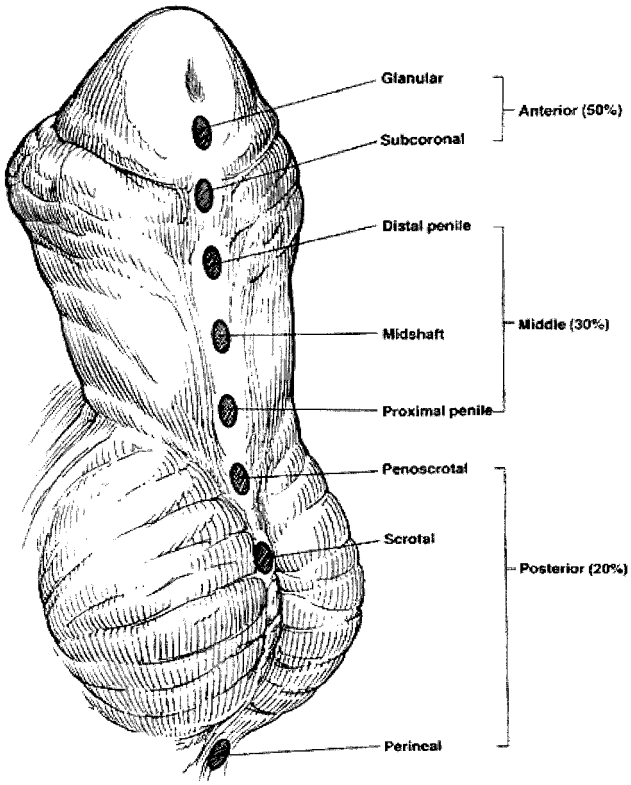

Figure 5. Schematic of different degrees of severity of hypospadias. Anterior hypospadias is also referred to as first-degree, middle as second-degree, and posterior as third-degree.

Undescended testes (cryptorchidism) and inguinal hernias are urogenital anomalies that are most commonly associated with hypospadias. In one series, 9.3% of hypospadias patients had undescended testes (Khuri et al., 1981). There was a 5% incidence of undescended testes with first-degree, 6% with second-degree, and 32% with third-degree hypospadias. The overall occurrence of inguinal hernia associated with hypospadias is estimated to be 9%, but with third degree hypospadias the occurrence can be as high as 17% (Khuri et al., 1981). Congenital anomalies not involving the urogenital tract have been found in 6.7% of all patients with hypospadias, and primarily involve the craniofacial, cardiothoracic, and gastrointestinal systems and the extremities. With increasing severity of hypospadias, the occurrence of associated non-urogenital anomalies can reach 12.7% (Latifoglu et al., 1998).

Epidemiology of Hypospadias

Hypospadias is a relatively common congenital anomaly, with a birth prevalence ranging from 0.3% to 0.8% of Caucasian male live births in the United States, and 0.05% to 0.4% for other racial groups (Bingol and Wasserman, 1990). A greater frequency of hypospadias has uniformly been found in Caucasians than in other races (Chavez et al., 1988; Gallentine et al., 2001). In the 1970s and 1980s, birth defect surveillance systems reported transient 1.5- to 2-fold increases in the prevalence of hypospadias in Norway (Bjerkedal and Bakketeig, 1975), Sweden (Kallen and Winberg, 1982), Denmark (Kallen et al., 1986), England (Matlai and Beral, 1985), and Hungary (Czeizel, 1985).

An international study of hypospadias from seven birth defect surveillance programs (Denmark, Hungary, Italy, Mexico, South America, Spain and Sweden) included data from over 7,400 cases of nonsyndromic hypospadias (Kallen et al., 1986). Differences in prevalence rates between countries were found that could not be explained completely by variations in case definition or different levels of ascertainment. The inclusion of less severe forms of hypospadias did not explain the higher rates in some programs compared to others. In some countries there was a 5–21% level of overascertainment due to misdiagnosis of normal infants. Counteracting this was a 30–64% underascertainment among cases severe enough to require surgery. However, geographic variability in prevalence rates could not be explained solely on the basis of ascertainment problems. Rates based on Swedish registry data from 1973–1974 (incomplete ascertainment) were 40% higher than rates from 1965–1968 based on hospital records and registry data (more complete ascertainment) (Kallen and Winberg, 1982). Consequently, hypospadias is difficult to assess in studies utilizing data from geographically diverse surveillance programs.

A Centers for Disease Control (CDC) study evaluated the birth prevalence of hypospadias in the United States from two birth defects surveillance systems: the Metropolitan Atlanta Congenital Defects Program (MACDP) and the Birth Defects Monitoring Program (BDMP) (Paulozzi et al., 1997). The MACDP is a hospital-based registry that was initiated in 1968 and is based on case ascertainment in 22 hospitals and clinics in the Atlanta, Georgia area. The BDMP is a population-based registry that was initiated in 1970, in which diagnoses from newborn discharge summaries are collected from a nationwide sample of hospitals. The total hypospadias rate (mild and severe) doubled in the MACDP between 1968 and 1993 from approximately 1.5 to 3.0/1000 births, and the overall trend was statistically significant. The annual rate of increase was 2.9%, and the overall increase occurred at a rate of 1.4% per year among Caucasians and 5.7% per year among non-Caucasians. For severe hypospadias, the rate increased three- to five-fold (0.11–0.55/1000 births) from 1968 to 1993, and this trend was also statistically significant. Overall, however, approximately 60% of cases could not be classified by severity. In the BDMP, the total hypospadias rate also doubled (2.02 to 3.97/1000 births) from 1970 to 1993, and this trend was also statistically significant. The increase occurred nationwide, and was highest in the Southeast and lowest in the West.

The prevalence of hypospadias was assessed in The Netherlands by prospective examination of newborns in Rotterdam over a 2-year period. A total of 7292 consecutive male births were examined for the presence of hypospadias (excluding glandular cases; see Fig. 5), and rates were increased four-fold compared to earlier time periods studied (Pierik et al., 2002). Consequently, comparable rates of increases in the birth prevalence of hypospadias have been seen in both the United States as well as in The Netherlands in studies utilizing population-based as well as hospital-based epidemiologic approaches. Recommendations have been made to investigate possible causes, including assessment of endocrine disrupting chemical (EDC) exposure (Dolk, 1998).

Risk Factors for Hypospadias

Several studies have found that male infants with hypospadias have lower birth weight, shorter length of gestation and/or evidence of growth retardation in utero (Sweet et al., 1974; Kallen and Winberg, 1982; Akre et al., 1999; Hussain et al., 2002). The recent study by Hussain et al. (2002) contains a systematic evaluation of all these risk factors. A retrospective cohort study of 6,746 male infants admitted to neonatal intensive care units (NICU) at the University of Connecticut from 1987–2000 was conducted. Overall, 1.66% of male infants had a diagnosis of nonsyndromic hypospadias, and there was a 10-fold increase in the birth prevalence over the 13-year period of the study. Hypospadias was significantly more common in infants who had poor intrauterine growth (<10th percentile) as measured by birth weight, length, and head circumference. The proportionate decrease in all of these growth parameters suggests that the primary insult occurred early, during the first trimester of pregnancy. There were no differences between singletons and multiple-gestation births, but the frequency was significantly higher among first-born infants (1.9%) compared to all other infants (0.9%). There was a higher occurrence (35%) of the more severe forms of hypospadias (second- and third-degree) in this study compared with other studies (13%: Avellan, 1975), indicating that both the incidence and severity were increasing over time. Common environmental factors that had an impact on intrauterine growth and morphogenesis of the genital tract were considered to be the cause. Exposure to EDC was implicated, as significant levels were found in aquatic life in proximity to the study site in Connecticut. While these findings are notable, and diagnostic criteria for hypospadias were rigorously controlled, the prevalence of hypospadias among infants in an NICU environment cannot be extrapolated to the general population.

An additional risk factor for hypospadias is parental subfertility, which has been identified as delayed childbearing in several studies. A 50% increase in severe cases has been demonstrated in children of mothers >35 years old compared to mothers <20 years old (Fisch et al., 2001). A correlation between paternal subfertility and increased risk of hypospadias has also been found. Abnormalities of the scrotum or testes (e.g., cryptorchidism, varicele, hydrocele, and atrophic testes) were found in 34% of index fathers whose sons had hypospadias compared to 3% of control fathers in a study conducted by Sweet et al. (1974). Fritz and Czeizel (1996) found that 24% of fathers with affected sons had signs of subfertility (such as decreases in sperm density, motility, and morphology) that required medical treatment, compared to 6% of controls. Subfertile males are now reproducing at a higher rate due to improvements in assisted-reproduction techniques, and this may be contributing to the increased occurrence of hypospadias (Czeizel, 1985; Fritz and Czeizel, 1996).

Additional evidence that paternal subfertility is a risk factor for hypospadias has come from studies of birth outcome following in vitro fertilization (IVF) procedures. A five-fold increased risk for hypospadias has been found in infants conceived by IVF procedures in the Greater Baltimore Medical Center as compared to statewide incidence figures in Maryland (Silver et al., 1999). These findings have been confirmed in subsequent studies using the Swedish Medical Birth Registry. No increased risk for hypospadias was found after standard IVF in Sweden; however, there was an approximately three-fold increased risk (95% CI, 1.4–5.4) after intracytoplasmic sperm injection (ICSI), a specific IVF procedure that is undertaken when sperm quality is poor (Wennerholm et al., 2000; Ericson and Kallen, 2001).

Women undergoing IVF procedures typically receive treatment with progesterone in the first trimester to support the pregnancy after embryo transfer. Silver et al. (1999) postulated that this treatment is the cause of increased risk for hypospadias following IVF procedures. Experimental studies have shown that progestins administered to laboratory animals during pregnancy can cause hypospadias (Goldman and Bongiovanni, 1967; Dean and Winter, 1984). The epidemiologic evidence, however, does not support an association between increased risk for hypospadias and first trimester exposure to progestins found in oral contraceptives, hormones for pregnancy support, and/or hormone-based pregnancy tests (Sweet et al., 1974; Aarskog, 1979; Czeizel et al., 1979; Mau, 1981; Monteleone et al., 1981; Calzolari et al., 1989; Kallen et al., 1991). A meta-analysis of human studies also did not find an association between progestin exposures and external genital anomalies in male infants (summary OR, 1.09; 95% CI, 0.90–1.32) (Raman-Wilms et al., 1995). In addition, Wennerholm et al. (2000) and Ericson and Kallen (2001) found in the Swedish Medical Birth Registry that an increased risk for hypospadias was specific for ICSI, and not for all the other IVF procedures in which progestin support was administered. These investigators concluded that confounding by paternal subfertility explained the association between ICSI and increased risk for hypospadias. The transfer of genes involving androgen action and metabolism from fathers with poor sperm quality to their sons was considered the most likely cause.

Genetic Predisposition for Hypospadias

There is a well recognized familial clustering of hypospadias, and male relatives of boys with hypospadias are more likely to have this condition than would be expected by chance. In a study by Sorenson (1953), male relatives of 103 index cases with nonsyndromic hypospadias were evaluated. It was found that 28% had at least one other family member with hypospadias. The more severe the hypospadias in the index case, the higher the incidence of hypospadias in first-degree relatives. With the mildest form of hypospadias, 3.5% of relatives were affected; with second-degree, 9.1%; and with third-degree, 16.7%. The overall risk for a brother of an affected infant to also have hypospadias was 9.6%. Chen and Woolley (1971) identified a similar figure of 9.7%. The risk of hypospadias in sibs of affected infants was found to be 4.2% in the international study, with a range of 0–11.3% (Kallen et al., 1986). These sib occurrence risks are compatible with a multifactorial mode of inheritance for hypospadias.

In a survey of 307 cases of familial hypospadias by Bauer et al. (1981), 25% of families had a second family member (in addition to the index child) with this anomaly, and 7% had three affected members. The risk of a second male sibling being born with hypospadias when this anomaly occurred for the first time in the index child was 11%. No boy with first-degree hypospadias had an affected brother. When the index child had a second- or third-degree hypospadias, a 12–19% chance existed, respectively, for hypo-

spadias to occur in an additional male sibling. An increased risk for hypospadias among twins has been described (Kallen et al., 1986). The prevalence of hypospadias is higher among members of male–male pairs and lower among males in male–female pairs. Concordance among twins of the same sex was 18% for both mild and severe forms, with increased risk evident in both monozygotic and dizygotic twins. When monozygotic twins discordant for hypospadias were evaluated, the twin with the lowest birth weight had hypospadias in 16 out of 18 pairs, suggesting a gene-environment interaction (Fredell et al., 1998).

Pedigree data do not suggest a Mendelian pattern of inheritance, and a multifactorial pattern is the most consistent explanation for familial clustering of severe hypospadias. Multifactorial models have yielded heritability indices ranging from 57% to 74% from pedigree data, indicating multifactorial causation with "high heritability" (Harris, 1990; Stoll et al., 1990). Allelic variants in genes involved in androgen action and metabolism may predispose individuals to develop hypospadias. When genetic susceptibility is combined with exposure to antiandrogenic agents, however, gene and environment risk factors may interact to surpass a threshold, resulting in occurrence of this birth defect.

Susceptibility Genes for Hypospadias

Steroid 5-alpha reductase type 2 (SRD5A2) (Genbank #L03843). The differentiation of the male external genitalia (penis, scrotum and urethra) depends upon conversion of testosterone (T) to dihydrotestosterone (DHT) via the enzyme steroid 5 alpha-reductase (SRD5A) located in the urogenital tubercle. DHT also initiates differentiation of the urogenital sinus into the prostate while it inhibits the development of the vesico-vaginal septum. T and DHT bind to the same intracellular androgen receptor and interact with a cognate DNA response element to regulate gene expression. Even though T and DHT are active via the same androgen receptor, these hormones produce distinct biological responses. The reasons for this are not clear at the molecular level; however, DHT binds to the androgen receptor more avidly than T, and the DHT-receptor complex is more efficiently transformed to the DNA-binding state than is the T-receptor complex (Siiteri and Wilson, 1974; Russell and Wilson, 1994; Zhu et al., 1998).

The role of T and DHT in formation of the male genital tract is vividly illustrated in men with congenital SRD5A enzyme deficiency, who are known as male pseudohermaphrodites (Griffin and Wilson, 1989; Imperato-McGinley et al., 1991; Zhu et al., 1998). They have a normal XY karyotype but ambiguous external genitalia, and thus are reared as females in many cases. T and estrogen (E) levels are normal to elevated, but DHT levels are reduced. These individuals lack Mullerian duct derivatives, while T-dependent Wolffian duct derivatives (the epididymides, seminal vesicles, vas deferens) are present. The DHT-dependent external genitalia are abnormal, however, and a small phallus, bifid scrotum, blind vaginal pouch, and varying degrees of hypospadias are present. At puberty, male pseudohermaphrodites develop a male habitus with male muscular development, deepening of the voice, enlargement of the phallus, and production of semen, but they have a hypoplastic prostate and scanty beard. Women with this genetic disorder are phenotypically normal and have no impairments of reproductive function.

Finasteride is a pharmacologic inhibitor of the SRD5A enzyme and selectively inhibits conversion of T to DHT; this drug is marketed for treatment of benign prostatic hyperplasia (PROSCAR®) and male pattern hair loss (PROPECIA®) (Merck & Co., Rahway, NJ). In rats, in utero exposure to low doses of finasteride results in decreased anogenital distance, transient production of nipples, hypospadias, and decreased prostate weight in male offspring (Clark et al., 1990, 1993). The critical period for these effects is gestation day (GD) 16–17 in the rat, following initiation of T synthesis by the fetal testes on GD 15. Additional studies have been carried out in rhesus monkeys (Prahalada et al., 1997). Oral administration of the drug from GD20–100 resulted in male genital tract abnormalities consisting of hypospadias, preputial adhesions to the glans penis, underdeveloped scrotum, and small penis. No other abnormalities were seen in male fetuses, and female fetuses were phenotypically normal. Defects of the male external genitalia produced by this pharmacologic SRD5A enzyme inhibitor are consistent with those seen in human male pseudohermaphrodites with a congenital deficiency of this enzyme.

Two different isozymes of SRD5A have been identified in humans: one is located in genital tissue and the prostate, with an optimum pH of 5.5 (type 2; SRD5A2), and the other is found in nongenital skin and the liver, with an optimal pH of 6–9 (type 1; SRD5A1) (Moore and Wilson, 1976). Male pseudohermaphrodites have deficiencies only in the SRD5A2 enzyme, the gene for which is located on the short arm of chromosome 2 (2p23). The gene for SRD5A1 is located on the short arm of chromosome 5 (5p15), and is normal in male pseudohermaphrodites (Andersson et al., 1991; Thigpen et al., 1992). In children, SRD5A1 expression is localized to the sebaceous gland in nongenital skin and is markedly elevated at puberty (Thigpen et al., 1993). The virilization of male pseudohermaphrodites at puberty may be influenced by synthesis of DHT in nongenital skin via SRD5A1 activity.

As a means of diagnosing SRD5A2 deficiency in children, T/DHT ratios have been measured in serum after hCG stimulation. Excretion of $5\alpha/5\beta$ steroid metabolites, and enzyme activity in cultured fibroblasts from genital skin have also been examined (Peterson et al., 1985). These parameters are highly variable and difficult to interpret in prepubertal children, and T/DHT ratios are increased only in the most severely affected patients (Hiort et al., 1996; Sinnecker et al., 1996). More recently, direct evaluation of allelic variants in the SRD5A2 gene has been used to diagnose SRD5A2 deficiency.

The SRD5A2 gene consists of five exons and four introns, spans over 40 kb of genomic DNA, and encodes a protein of 254 amino acids (Labrie et al., 1992; Thigpen et al., 1992). Mutations in the SRD5A2 gene have been associated with male pseudohermaphrodism in isolated kindreds (Hochberg et al., 1996). This syndrome was first described at the clinical and molecular level in 1974 (Imperato-McGinley et al., 1974; Walsh et al., 1974), and a number of other individual cases and consanguineous kindreds have since been identified, as described in Table 1. Allelic variants have been found throughout the SRD5A2 gene in these subjects, including deletions, missense and nonsense mutations, splicing defects, and frameshift mutations. Approximately

Table 1
Summary of Allelic Variants in the SRD5A2 Gene of Male Pseudohermaphrodites

Location	Type	Comment	Reference
Deletion	Deletion all exons	Inactivates enzyme	Andersson et al., 1991
Exon 1			
G34R	Missense	Reduced activity	Johnson et al., 1986
G37R	Missense	Not determined	Canto et al., 1997
L55Q	Missense	Inactivates enzyme	Hochberg et al., 1996
Q56R	Missense	Inactivates enzyme	Peterson et al., 1985
G85D	Missense	Not determined	Vilchis et al., 2000
Y91D	Missense	Inactivates enzyme	Russell and Wilson, 1994
Exon 2			
R103	Premature termination	Inactivates enzyme	Zhu et al., 1998
I112N	Missense	Reduced activity	Sinnecker et al., 1996
G115D	Missense	Inactivates enzyme	Cai et al., 1996
Q126R	Missense	Inactivates enzyme	Thigpen et al., 1992
E126R	Missense	Not determined	Ferraz et al., 1999
DelT140	Frameshift	Not determined	Ferraz et al., 1999
R145W	Missense	Inactivates enzyme	Zhu et al., 1998
Exon 3			
DelMet157	Deletion	Reduced activity	Boudon et al., 1995
D164V	Missense	Inactivates enzyme	Thigpen et al., 1992
R171S	Missense	Reduced activity	Jenkins et al., 1992
P181L	Missense	Inactivates enzyme	Russell and Wilson, 1994
G183S	Missense	Reduced activity	Cai et al., 1996
Exon 4			
N193S	Missense	Reduced activity	Russell and Wilson, 1994
G196S	Missense	Reduced activity	Wilson et al., 1993
E197D	Missense	Inactivates enzyme	Chavez et al., 2000
E200K	Missense	Not determined	Anwar et al., 1997
G203S	Missense	Not determined	Canto et al., 1997
A207D	Missense	Inactivates enzyme	Canto et al., 1997
P212R	Missense	Inactivates enzyme	Chavez et al., 2000
L224P	Missense	Inactivates enzyme	Canto et al., 1997
R227stop	Premature termination	Inactivates enzyme	Vilchis et al., 2000
R227G	Missense	Reduced activity	Hiort et al., 1996
A228T	Missense	Reduced activity	Sinnecker et al., 1996
H230P	Missense	Inactivates enzyme	Russell and Wilson, 1994
H231R	Missense	Reduced activity	Boudon et al., 1995
Ex 4-In 4	Splice junction	Inactivates enzyme	Thigpen et al., 1992
Exon 5			
Y235F	Missense	Not determined	Zhu et al., 1998
R246W	Missense	Inactivates enzyme	Thigpen et al., 1992
R246Q	Missense	Inactivates enzyme	Jenkins et al., 1992
DelA251	Frameshift	Inactivates enzyme	Can et al., 1998

two-thirds of male pseudohermaphrodites are homozygous, and one-third are compound heterozygous for these mutations (Thigpen et al., 1992; Russell and Wilson, 1994). The functional significance of most allelic variants has been characterized. All reduce V_{max} of the enzyme, and many result in no detectable enzyme activity. Although individual allelic variants have been extensively characterized, it has not been possible to correlate the severity of male pseudohermaphrodism with specific alterations in the SRD5A2 gene. As most of the subjects studied to date have been from consanguineous families, it remains to be determined whether the mutations identified to date are representative of what would be seen in the general, outbred population of infants with nonsyndromic hypospadias.

The SRD5A2 gene has also been extensively studied in men with prostate cancer drawn from the general population, and the allelic variants identified are listed in Table 2. Findings from these studies are of interest in determining whether the allelic variants seen in the SRD5A2 gene of male pseudohermaphrodites (most of whom are from consanguineous families) are similar to those in prostate cancer patients drawn from the general population. Ten single nucleotide polymorphisms (SNPs) resulting in amino acid substitutions have been described by Makridakis et al. (2000), and a TA repeat polymorphism was found by Kantoff et al. (1997) in the 3′-untranslated region (3′-UTR) of the gene. Most of the allelic variants are rare, with allele frequencies <1.0%. An exception is the V89L substitution, which has an allele frequency of 32.5%. This common variant results in an approximately 60% decrease in enzyme activity and a corresponding decrease in circulating androstanediol glucuronide levels, an index of whole-body 5-alpha reductase activity (Reichardt et al., 1995; Febbo et al., 1999; Lunn et al., 1999). The V89L substitution has not been reported to date in male pseudohermaphrodites (Table 1), which indicates that the allelic variants in this cohort may not be representative of the general population, and may reflect consanguinity of the families studied.

Table 2
Summary of Allelic Variants in the SRD5A2 Gene in Prostate Cancer Studies

Location	Type (allele frequency)	Comment	Reference
Exon 1			
C5R	Neutral (1.2%)	No change activity	Makridakis et al., 2000
P30L	Missense (0.2%)	Decreased activity	
P48R	Missense (0.5%)	Decreased activity	
A49T	Missense (2.0%)	Increased activity	
A51T	Missense (0.2%)	Decreased activity	
V89L	Missense (32.5%)	Decreased activity	
Exon 4			Makridakis et al., 2000
T187M	Missense (0.3%)	Decreased activity	
F194L	Neutral (0.1%)	No change activity	
R227Q	Missense (0.5%)	Decreased activity	
Exon 5			
F234L	Missense (0.3%)	Decreased activity	Makridakis et al., 2000
3′UTR			
TA repeat	TA(0) (0.87%) TA(9) (0.13%) TA(18) (0.01%)	No change activity	Kantoff et al., 1997

17-beta hydroxysteroid dehydrogenase gene (HSD17B3; GenBank #605573). Different HSD17B isozymes (designated as types I–IV) have been found and named according to the chronological order in which their cDNAs were cloned (Penning, 1997). The HSD17B3 isozyme is found exclusively in the microsomes of the testes, where it converts the weak androgen, androstenedione (ΔA), into T with NADPH as a cofactor. The HSD17B3 gene spans at least 60 kb of DNA, contains 11 exons, is located on chromosome 9q22, and encodes a protein of 310 amino acids. The 1.3-kb mRNA encoding the HSD17B3 isozyme has been detected only in the testis, which is consistent with its role in the formation of testicular androgens (Geissler et al., 1994; Andersson, 1995).

Familial male pseudohermaphrodism due to an autosomal recessive deficiency in the HSD17B3 isozyme has been reported (Castro-Magana et al., 1993). The phenotype is similar to that of SRD5A2 deficiency and includes 46, XY individuals with undescended testes and normal Wolffian duct derivates (epididymides, vas deferens, and seminal vesicles), but with external female genitalia. Although typically they are raised as females, affected individuals develop male body habitus and male secondary sexual characteristics at puberty. The metabolism of ΔA to T, although deficient, may produce enough T for stabilization of the Wolffian ducts but not enough T to serve as a precursor for DHT, resulting in inadequate masculinization of the external genitalia (Rosler, 1992). Deficiency in HSD17B3 activity is rarely diagnosed at birth, and is usually discovered at puberty in genetic males reared as females. The symptoms that bring these "phenotypic" females to medical attention are the presence of gynecomastia, amenorrhea, clitoral enlargement, and hirsutism. A diagnosis of HSD17B3 deficiency at puberty is made on the basis of abnormally high ΔA, LH, and FSH levels, and subnormal to normal T and DHT levels (Kohn et al., 1985). Genetic females who are homozygous or heterozygous for mutations in this gene are phenotypically normal (Lundqvist et al., 2001).

A total of 20 allelic variants have now been described in the HSD17B3 gene in subjects with HSD17B3 isozyme deficiency (Table 3). Most of these variants cause complete loss of enzyme activity. Many of these mutations have been found in consanguineous families or small case series of individuals with a family history of genital ambiguity. As a result, most are present in homozygous form, and there are relatively few heterozygotes. The R80Q mutation has been found in 24 individuals from nine consanguineous Arab kindreds in Israel (Rosler et al., 1996). In addition, a homozygous mutation (G→A transition at the boundary between intron 8 and exon 9 that disrupts the splice acceptor site of exon 9) was found in the HSD17B3 gene of a male pseudohermaphrodite from a consanguineous Turkish kindred, with a high penetrance of SRD5A2 gene mutations (Can et al., 1998). Most of the male pseudohermaphrodites in this Turkish kindred were homozygous for either SRD5A2 or HSD17B3 gene defects; however, a few were homozygous for the SRD5A2 defect and heterozygous for the HSD17B3 defect, or homozygous for the HSD17B3 defect and heterozygous for the SRD5A2 defect. Male pseudohermaphrodites with homozygous SRD5A2 or HSD17B3 gene defects were phenotypically distinguishable by the presence of gynecomastia in the latter.

In a nationwide survey of male pseudohermaphrodites in The Netherlands, 18 index cases and two siblings with HSD17B3 mutations were found (Boehmer et al., 1999). Gonadotropin-stimulated T/ΔA ratios were discriminative in all cases, and did not overlap with ratios in normal controls or in patients with androgen insensitivity syndrome (AIS). In all patients, both HSD17B3 alleles were mutated. The minimal incidence of 17BHSD3 deficiencies in The Netherlands was shown to be 1:147,000, with a heterozygote frequency of 1:135. At least four mutations (325+4 A→T, N74T, 655–1 G→A and R80Q) were found worldwide, as well as in The Netherlands. These mutations apparently originated from genetic founders whose dispersion could be reconstructed from ancient historical migrations. The 326-1 G→C and P282L were de novo mutations that were found to be recurrent in different populations. From these findings, it is anticipated that mutations in the HSD17B3 gene will be found to have an independent association with hypospadias. It is also possible that allelic variants in the HSD17B3 gene will occur simultaneously with variants in the SRD5A2 gene, as dem-

Table 3
Summary of Allelic Variants in the HSD17B3 Gene of Male Pseudohermaphrodites

Location	Type	Comment	Reference
Exon 2			
A56T	Missense	Residual enzyme activity	Moghrabi et al., 1998
S65L	Missense	Inactivates enzyme	Andersson et al., 1996
Exon 3			
N74T	Missense	Residual enzyme activity	Boehmer et al., 1999
R80Q	Missense	Residual enzyme activity	Geissler et al., 1994
R80W	Missense	Residual enzyme activity	Bilbao et al., 1998
N130S	Missense	Residual enzyme activity	Bilbao et al., 1998
Intron 3			
325 + 4, A → T	Splice junction	Disrupts splice donor	Andersson et al., 1996
326-1, G → C	Splice junction	Disrupts splice acceptor	Geissler et al., 1994
Exon 8			
Q176P	Missense	Inactivates enzyme	Moghrabi et al., 1998
Intron 8			
655-1, G → A	Splice junction	Disrupts splice acceptor	Geissler et al., 1994
Exon 9			
A203V	Missense	Inactivates enzyme	Geissler et al., 1994
V205E	Missense	Inactivates enzyme	Andersson et al., 1996
F208I	Missense	Inactivates enzyme	Andersson et al., 1996
E215D	Missense	Inactivates enzyme	Andersson et al., 1996
Exon 10			
Δ777 → 783	Deletion	Frameshift, truncates enzyme	Andersson et al., 1996
S232L	Missense	Inactivates enzyme	Geissler et al., 1994
C268Y	Missense	Inactivates enzyme	Lundqvist et al., 2001
Exon 11			
M235V	Missense	Inactivates enzyme	Andersson et al., 1996
P282L	Missense	Inactivates enzyme	Andersson et al., 1996
G289S	Neutral	No change activity	Boehmer et al., 1999

onstrated in the Turkish kindred, resulting in a polygenic effect.

Androgen receptor gene (AR; GenBank #M21748). The AR gene plays a critical role in male sexual differentiation by mediating the biological effects of gonadal androgens. The AR gene resides on Xq11-12 and consists of eight exons. Exon 1 contains a highly polymorphic CAG repeat (range 11–31 CAGs) coding for a polyglutamine tract. Functional studies with different CAG repeat numbers have indicated an inverse relationship between the CAG repeat length and expression level of the AR gene (Hakimi et al., 1997). Reduction in the number of CAG repeats results in increased expression levels of the AR gene, and thus increased risk for severe and early-onset prostate cancer (Giovannucci et al., 1997; Ross et al., 1998). Expansion in the number of repeats has been associated with decreased AR transcription factor activity and thus increased risk for low virilization, reduced sperm production, testicular atrophy, and infertility (MacLean et al., 1995; Tut et al., 1997; Dowsing et al., 1999; Dadze et al., 2000).

There have been several conflicting studies on the relationship between CAG repeats and male sexual differentiation. Significant expansions of CAG repeat length have been reported in patients with moderate to severe undermasculinization, including micropenis with hypospadias or genital ambiguity (Lim et al., 2000). Ishii et al. (2001) did not find any expansion in patients with isolated micropenis, and neither did Muroya et al. (2001) in patients with isolated hypospadias. However, these studies had relatively few patients, and there is a rationale for examining CAG repeat length in larger studies, given that paternal subfertility and poor semen quality are established risk factors for hypospadias.

There are more than 150 mutations described in the AR gene that give rise to androgen insensitivity syndrome (MacLean et al., 1995). Most reports of AR gene mutations in individuals with hypospadias have included patients with additional genitourinary malformations, and the disorder analyzed was partial androgen insensitivity syndrome rather than nonsyndromic hypospadias. Mutations of the androgen receptor coding sequence are infrequent in patients with isolated hypospadias (Allera et al., 1995; Klocker et al., 1995; Sutherland et al., 1996).

In a study of 63 cases of severe hypospadias obtained from a single center (Boehmer et al., 2001b), cases were evaluated for a spectrum of known risk factors, including patient history, physical examination, karyotyping, hormonal evaluation, and assay of genes involved in androgen action and metabolism. In 31% of cases, the underlying etiology was identified. Of these, 17% were due to complex genetic syndromes, such as Smith-Lemli-Opitz and Opitz-Frias. Sex chromosomal anomalies, such as XY mosaicisms, accounted for 9.5% of cases. Isolated causes in one case each were the androgen insensitivity syndrome and 5α-reductase type 2 deficiency. In patients with decreased T to ΔA ratios, no mutations were found in the HSD17B3 gene. Also, in patients with elevated T to DHT ratios, no mutations were found in the SRD5A2 gene. These findings indicate that gene mutations in the SRD5A2, HSD17B3, and AR genes may not be common in patients drawn from the general population with nonsyndromic hypospadias. Until larger studies are undertaken, however, the role of genetic factors in genes controlling androgen action and

metabolism will not be clearly understood. Evaluations of genotype, as well as exposure to endocrine disrupting chemicals, are likely to be important in explaining the majority of cases of nonsyndromic hypospadias.

Endocrine Disrupting Chemicals

Rates of hypospadias have increased in parallel with other disorders of the male reproductive tract, including testicular cancers, cryptorchidism, and poor semen quality. Prener et al. (1996) found that testicular cancer risk increases in patients with previous cryptorchidism (RR, 5.2; 95% CI, 2.1–13.0), inguinal hernia (RR, 1.8; 95% CI, 0.9–3.7), hypospadias (RR, 4.2; 95% CI, 0.4–42.7), and hydrocele (RR, 2.4; 95% CI, 0.6–9.0). These results add to the growing body of evidence that common genetic and environmental risk factors have a role in male reproductive tract disorders. The familial clustering of hypospadias among first-degree relatives has traditionally been perceived as evidence of a genetic component in the etiology of this anomaly. However, exposure to environmental contaminants is now being considered in familial clusters because of the high probability of shared exposures among first-degree relatives (Baskin et al., 2001).

Several environmental antiandrogens have been identified in rodent models that interfere with male sexual differentiation at environmentally relevant doses. At relatively low in utero doses, antiandrogens reduce anogenital distance and induce transient nipple development in the neonatal rat. At mid-doses, hypospadias, agenesis of the sex accessory tissues, and retained nipples occur, while at the highest doses undescended testes and epididymal agenesis are seen. Fetal tissue concentrations of 10–20 ppm of the DDT metabolite, p,p′-DDE, an AR antagonist, are sufficient to produce these antiandrogenic effects in the rat fetus (Kelce et al., 1995). These concentrations are similar to those measured in first-trimester human fetal tissues in the late 1960s. The pesticides vinclozolin, procymidone, linuron, and fenitrothion are also AR antagonists that produce dose-response effects on the developing male reproductive system (Gray et al., 1999a; Tamura et al., 2001). Phthalate esters (PE) inhibit testosterone synthesis during fetal life, and produce dose-related abnormalities of the male reproductive tract similar to those caused by the AR antagonists (Gray et al., 2000). The PE have effects at extremely low in utero doses, and no-effect levels could not be found for the most sensitive endpoint, reduction in anogenital distance in male neonates. Prenatal administration of a single low dose of dioxin (50–1,000 ng TCDD/kg) alters differentiation of androgen-dependent tissues; however, the mechanism of action involves interaction with the hormone-like receptor, AhR, rather than the AR (Gray et al., 2001). Attempts to extrapolate findings from these rodent models to humans have been problematic. It can be difficult to determine whether effects obtained at doses employed in rodent models are relevant to human environmental exposures. An even greater problem is the difficulty of accurately measuring in utero human exposure to environmental agents for comparison.

Results from human studies have cast doubt on whether findings of male reproductive-tract abnormalities in rodents with low doses of p,p′-DDE are meaningful for risk assessment. Longnecker et al. (2002) analyzed stored maternal serum samples from the Collaborative Perinatal Project that had been collected in 1959–1966, a time when DDT exposures of human populations were high. They examined the relationship between maternal DDE levels during pregnancy from these samples, and risks for cryptorchidism (n = 219), hypospadias (n = 199), and extra nipples (n = 167) among male offspring. Compared to boys whose mother's serum p,p′-DDE level was <21.4 μg/liter, boys with maternal levels ≥85.6 μg/liter had adjusted ORs of 1.3 (95% CI, 0.7, 2.4) for cryptorchidism, 1.2 (95% CI, 0.6, 2.4) for hypospadias, and 1.9 (95% CI, 0.9, 4.0) for extra nipples. The authors concluded that there may be a modest association between exposure to p,p′-DDE and these reproductive tract abnormalities, but that the results were inconclusive.

The effects of "estrogenic" environmental agents on male reproductive-tract development have been well studied in laboratory animals and humans. Male rat pups exposed to DES (0.015–0.60 mg/kg sc) in mid-gestation have hypospadias at all treatment levels (Vorherr et al., 1979). However, men exposed to DES in utero have a broad range of reproductive-tract problems (e.g., difficulty voiding and abnormalities of the urethra), but increased risk for hypospadias has not been observed in this cohort (Henderson et al., 1976). DES and estradiol have a high affinity for the estrogen receptor as well as for the AR gene, and their effects on male reproductive-tract development may be mediated via antiandrogenic properties as well as estrogenic activity. Relatively few environmental "estrogens" have been evaluated for effects on both the estrogen and androgen receptors, with the exception of DDT and methoxychlor. One metabolite of DDT, o,p′-DDT, has weak estrogenic activity while another, p,p′-DDT, is an AR antagonist (Kelce et al., 1995). Active metabolites of methoxychlor bind to both the estrogen and androgen receptors, resulting in both estrogenic and antiandrogenic effects, depending on the time of exposure and target tissue involved (Gray et al., 1999b).

The effects of maternal diet, particularly vegetarianism and consumption of phytoestrogens, on the risk for hypospadias have been examined in a longitudinal study with approximately 8,000 subjects in the United Kingdom (North and Golding, 2000). Among a broad spectrum of risk factors evaluated, mothers who were vegetarian during pregnancy had an adjusted OR of 4.99 (95% CI, 2.10–11.9) for having an affected son compared to mothers who were ominivores and did not supplement their diet with iron. The only other statistically significant association for hypospadias was influenza in the first 3 months of pregnancy (adjusted OR, 3.19; 95% CI, 1.50–6.78). Mothers who were vegetarians before pregnancy but omnivores during pregnancy had no increased risk for having a son with hypospadias, compared to mothers who were never vegetarians. The risks of a vegetarian diet in this study appeared to be related to consumption of soy products, as vegetarian mothers who consumed only organic produce (fruits and vegetables) had no excess risk for having a son with hypospadias.

A finding of concern has been the effects of low doses of atrazine, the most commonly used herbicide in the United States, on sexual development of frogs (*Xenopus laevis*) (Hayes et al., 2002). Larvae were exposed to atrazine (0.01–200 ppb) by immersion throughout larval development, and gonadal histology and laryngeal development were examined at metamorphosis. Atrazine (≥0.1 ppb) induced hermaphroditism and demasculinized the larynges of ex-

posed males via a reduction in circulating T levels. The authors hypothesized that the mechanism of action was induction of aromatase activity, which would promote conversion of T to E and produce antiandrogenic effects (demasculinization of larynges) and estrogenic effects (hermaphroditism). No previous studies have addressed the effects of atrazine at concentrations below those considered safe in drinking water (3 ppb) or safe for limited human exposure (200 ppb). The effects on gonads in male frogs were produced at 0.1 ppb, which is more than 600 times lower than the dose required to induce aromatase in human adrenocortical and placental tissues, and 30,000,000 times lower than the dose required to produce reproductive effects in rats. The extreme sensitivity of *Xenopus* to perturbation of male sexual differentiation by atrazine is remarkable, and relevant for these amphibians in the wild, but is of uncertain relevance for human health effects.

A number of epidemiology studies have been conducted concerning pesticide exposures of fathers employed as gardeners, agricultural pilots, or aerial sprayers. These studies have provided weak evidence, if any, that these types of occupational exposures result in adverse pregnancy outcomes (Smith et al., 1981, 1982; Roan et al., 1984; Rupa et al., 1991). A review of epidemiology studies published in 1980–1996 on the effects of agricultural occupation (and presumably pesticide exposure) on congenital malformations has been conducted (Garcia, 1998). Of 34 published studies, few resulted in significant associations between birth defects and pesticide exposure. Problems inherent in most of the studies were the use of occupational title or residence as a surrogate for pesticide exposure, the assessment of outcome through parental interview rather than medical records, and small study size. With few exceptions, the estimates for association between agricultural work and/or pesticide exposure and congenital malformations were below 2.0. Recommendations from this and other reviews are that exposure to specific active ingredients or at least chemical classes must be quantitatively evaluated before assessment of effects on reproductive function can be accurately determined (Nurimen, 1995). Regardless of these methodological issues, however, enough positive associations have been reported to warrant further investigation of pesticide effects on human reproduction.

CONCLUSIONS AND RECOMMENDATIONS

Evidence for increases in the birth prevalence of hypospadias in the United States is substantial. Multiple studies utilizing population-based, as well as hospital-based, approaches have documented increases in both mild and severe forms of hypospadias from the 1960s to the present. Several clinical risk factors that have been identified, including intrauterine growth reduction and paternal subfertility, particularly when accompanied by assisted-reproduction procedures. Familial clustering has been well documented, and may involve both genetic and environmental risk factors. A number of candidate genes that control androgen action and metabolism are logical choices for genomic evaluation in population-based studies, including the SRD5A2, HSD17B3, and the AR genes. In consanguineous families, reduction/loss of function mutations in these genes have been found to be causative for male pseudohermaphrodism. While a number of other gene pathways have been implicated (e.g., HOX, FGF, and SHH [see Baskin et al., 2001 for review]), it is likely these pathways would be more important in multiple malformation syndromes including hypospadias than in isolated hypospadias. The role of EDC exposure in the etiology of hypospadias in human populations is far from clear. Major improvements in methodologies to measure environmental exposures and pregnancy outcome are needed to determine the role of environmental exposures in the increased rates of hypospadias.

Studies that simultaneously examine the roles of allelic variants in genes controlling androgen action and metabolism, and environmental exposures are needed to elucidate the risk factors for nonsyndromic hypospadias. The rationale for such studies is that the risk from environmental exposures may only be evident in genetically susceptible subgroups, and the risk of a genotype may only be evident in exposed populations. There has not yet been a study in a large, outbred population of infants with hypospadias to determine whether allelic variants in genes controlling androgen action and metabolism are associated with hypospadias. Such an association study would improve our understanding of the genetic basis for hypospadias in the general population.

Allelic variants in genes can be measured with far greater precision compared to environmental exposures. For this reason alone, the probability of identifying genetic risk factors for hypospadias is greater than for environmental exposures. Despite the potential for environmental exposure misclassification, and the possible loss of power in estimating interaction effects, gene by environment interaction studies must be undertaken before we can begin to understand the risk factors for this rapidly increasing birth defect.

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