Chapter 1

Introduction
1.1 Sex-determination

Sexual reproduction is the complex process, which ascertains the development of a perfect new organism as per the biological message contributed by egg and sperm from the parents. The single cell (zygote) ultimately develops into the tissues, organs and organ systems, everything perfectly placed and controlled in time and space. The post-zygotic cells divide and differentiate into the destined organ according to their fates. Most of the organs follow the same developmental path in both the sexes of human. However, the indifferent gonad is the only organ with two different fates between male and female sex. The development of either testes in males or ovaries in females leads to further differentiation into that sex. Sex-determination is a complex yet poorly understood phenomenon. In human sex is determined at two levels; gametic and gonadal.

**Gametic sex-determination:** In humans males are heterogametic while females are homogametic. Therefore the gametic sex-determination depends upon the fusion of either Y or X bearing sperm with the egg.

**Gonadal sex-determination:** After the formation of the zygote, the process of the development starts with the process of sex-determination. The gonad differentiation starts around 7th week of pregnancy. The expression of the *SRY* (sex determining region on the Y chromosome) gene at 11.5 days post coitum (dpc) in genetically male mice starts the process of testis differentiation. The migration of the germ cells from the mesonephros and formation of the testicular cords is a crucial phase of the testis differentiation. The testis differentiation results in the formation of Sertoli cells inside the seminiferous tubules while the Leydig cells in the interstitial space between the seminiferous tubules (Figure 1.1).
Figure 1.1. Schematic view of the events in sex-determination and differentiation.
1.2 Sex-differentiation

The process of sex-differentiation follows gonad differentiation. The hormones produced by the differentiated gonads result in the overall secondary sexual differentiation (Figure 1.1). Leydig cells in the testes produce testosterone. Testosterone is transported to the target tissues via blood stream, where a part of it is converted to dihydrotestosterone (DHT) by the action of 5-α reductase. Testosterone and DHT carry out the male secondary sexual differentiation. Sertoli cells also produce anti-mullerian hormone, which results in the regression of mullerian ducts in the male individuals. The androgens produced by the Leydig cells are essential for the development of the wolffian ducts in males, which gives rise to the vas deferens, prostate and accessory ducts. Androgens are also essential for the changes which take place at puberty in males. On the other hand, it is thought that the estrogens are not required for female secondary sexual differentiation. The lack of the anti-mullerian hormone and the presence of estrogens at puberty ensure the differentiation of the mullerian ducts which gives rise to the uterus, fallopian tubes in females. Estrogens play role in female secondary sexual differentiation at puberty (Figure 1.1).

1.3 Sex reversal

The defect in the molecular signaling at any step of the sex-determination pathway may result in the development of the characteristics of the opposite sex, resulting in partial or complete sex reversal. The sex reversed individuals present with various combinations of gonad types and the external genitalia, depending upon the chromosome complement and

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the severity of the genetic defect. The genetic defect in the genes involved in sex-determination, usually result in gonadal dysgenesis while the defects in the genes involved in sex-differentiation result in the development of the either testis or ovaries depending upon the chromosome complement but results in development of secondary sexual characters of the opposite sex. It is largely the study of these individuals, which has helped understanding the process of sex-determination and differentiation. Sex reversal cases fall into various categories depending upon the genetic complement, type of the gonads and the overall phenotype.

1.3.1 Female Pseudohermaphroditis

By definition, a female pseudohermaphrodite is a genetically female (46,XX) with ovaries but virilized external genitalia. This may arise from either endogenous production of androgens or exogenous androgen exposure such as from a maternal source. The most common variant of classic ambiguous genitalia is the female pseudohermaphrodite secondary to congenital adrenal hyperplasia. Cholesterol is converted in adrenals into androgens, mineralocorticoids, and cortisol. The most common defect that leads to ambiguous genitalia and female pseudohermaphroditism is a defect in the enzyme 21-hydroxylase in the steroid biosynthesis pathway. Other enzymatic defects in the process may also lead to both male and female pseudohermaphroditism. Cortisol levels drive the process through feedback loops in the hypothalamus and pituitary. If cortisol levels are low because of an enzymatic defect preventing its production, such as when there is a 21-hydroxylase deficiency, then the pituitary responds by increased ACTH secretion leading to further stimulation of the system and a buildup of the precursor molecules. Such a shift
leads to overproduction of androgens leading to virilization and underproduction of the mineralocorticoids leading to salt wasting.

Patients with the uncommon 11β-hydroxylase deficiency will have both virilism and hypertension from the elevation of androgens and the elevation of the mineralocorticoid 11-deoxycorticosterone. Congenital adrenal hyperplasia (CAH) is alone among the causes of ambiguous genitalia as being potentially life threatening in the newborn, because of the potential for mineralocorticoid deficiency leading to hyponatremia, hyperkalemia, dehydration, and circulatory collapse during the 2nd week of life.

1.3.2 Male Pseudohermaphroditism

A male pseudohermaphrodite has a 46,XY karyotype but deficient masculinization of the external genitalia. There are three basic mechanisms of undervirilism that lead to male pseudohermaphroditism. It may occur due to inadequate testosterone production, inadequate testosterone metabolism (SRD5A2 deficiency), or through insensitivity to androgens due to androgen receptor (AR) defect.

Inadequate testosterone production may occur from Leydig cell deficiency, which may be due to testicular hypoplasia or absence. Testicular absence is thought to occur most commonly as testicular regression rather than primary agenesis. It is surmised that a fetal mishap such as bilateral prenatal torsion leads to loss of testicular tissue. More commonly, inadequate production of androgens is due to an inborn error in androgen biosynthesis. Examples of enzymatic defects which may lead to male pseudohermaphroditism are 3 β-hydroxysteroid dehydrogenase, 17-hydroxylase, 17, 20
desmolase, and 17 β-hydroxysteroid dehydrogenase deficiencies. The level of under-virilism varies in each of these conditions depending on the degree of enzymatic block, level of the block, and time of diagnosis. Some conditions such as 17 β-hydroxysteroid dehydrogenase deficiency are initially associated with nearly complete lack of virilization until puberty when they may present as severe late virilization in a phenotypic female (Forest, 2001).

The second cause of male pseudohermaphroditism is inadequate testosterone metabolism, which is secondary to 5α-reductase (SRD5A2) deficiency. Testosterone production is usually normal in these individuals but they are unable to convert it to dihydrotestosterone (DHT), which is responsible for virilization of the external genitalia. These patients have a 46,XY karyotype, regression of Müllerian ducts, normal Wolffian duct structures, hypoplastic prostates and varying degrees of undervirilism of the external genitalia. The typical patient is a phenotypic female at birth with normal male internal genitalia. Most are raised as females. At puberty, they will exhibit virilization, phallic growth, testicular descent and deepening of the voice. Since brain receptors respond to testosterone rather than DHT, these patients may have a male gender identity (Forest, 2001).

The third and the most common cause of male pseudohermaphroditism is inadequate response to the otherwise normal levels of androgens, because of androgen receptor defect. Androgen receptor defects may lead to complete or incomplete loss of function. The former condition is commonly referred to as testicular feminization syndrome. These patients often present at puberty as phenotypic females with amenorrhea, or they may present earlier as a phenotypic female with an inguinal hernia containing a palpable
Male genitals, infertility and/or undermasculinization in otherwise normal males

Male genitals, mildly under-masculinized, isolated hypospadias

Severely under-masculinized (undescended testes, and/or bifid scrotum)

Ambiguous genitals, severely under-masculinized, clitoromegaly

Female genitals (including separate urethral and vaginal orifices, mild clitoromegaly)

Female genitals with small labial folds, normal pubic/underarm hair

Female genitals with little or no pubic/underarm hair

Figure 1.2. Various grades of androgen insensitivity syndrome.
gonad, which turns out to be a testis (Figure 1.2). Often these patients have a normal female habitus, breast development, sparse pubic hair, and a short blind-ending vagina, which may be adequate for intercourse. They are sterile, but otherwise physically and mentally female and are always reared as such. The patients usually lack uterus and ovaries, however sometimes a rudimentary uterus may be present (Dapunt et al., 1975). Testosterone levels are usually elevated at the time of puberty with or without elevated levels of leutinizing hormone (LH). Elevated testosterone levels also serve as substrate for estrogen synthesis, which results in further feminization in CAIS patients (Quigley et al., 1995). Because of the risk of gonadal tumors in these individuals, testes should be removed.

Patients with partial androgen insensitivity present with incomplete masculinization of varying degree. More severe forms will resemble an overvirilized female more than an undervirilized male. The patients have predominantly female phenotype (female external genitalia, pubic hairs with or without clitoromegaly and partially to completely fused labia) to ambiguous genitalia to predominantly male phenotype with micropenis, perineal hypospadias and cryptorchidism (Quigley et al., 1995). The later group of the patients is also termed as Reifenstein syndrome (Quigley et al., 1995). PAIS patients are assigned a grade (Figure 1.2) according to the severity of androgen insensitivity and affinity of the phenotype with male or female pattern. Males with mild androgen insensitivity (MAIS) usually have normal male genitals and internal male structures and are normal at birth. However, during puberty they may have breast enlargement, sparse facial and body hair,
and small penis (Tsukada et al., 1994). Some affected males may also have impaired sperm production resulting in oligozoospermia or azoospermia (Yong et al., 2000).

A rare disorder called *hernia uteri inguinalis* is seen with inadequate Sertoli cell production of MIS, leading to persistent Müllerian ductal structures. These patients are normally masculinized; however, at the time of hernia or correction of undescended testis, a uterus and fallopian tube may be found.

1.3.3 *True Hermaphrodites*

The phenotype associated with true hermaphrodites varies between the extremes of female/male differentiation. However, the majority of patients show some signs of virilization. There are regional differences in the incidence, time of diagnosis, and karyotype of true hermaphrodites. While it is rare in the West, it is one of the more common types of intersex in Africa. Most children are diagnosed at birth because of partial virilization; however, in the Third World, late presentation is common. Regional variations in karyotype show that 46,XX is most common in Africa, mosaicism is more common (46,XX/46,XY and 47,XXX/46,XY) in Europe and North America, and 46,XY is common in Japan (MacLaughlin and Donahoe, 2004).

The hallmark of true hermaphroditism is the finding of gonads with both testicular and ovarian tissue; the latter being different from a streak gonad by the presence of a well-formed follicle. Ovarian tissue generally matures normally with follicular maturation at puberty. Testicular tissue by contrast, will often have progressive fibrosis with age and spermatogenesis is rare. Hormone production usually follows histologic changes. At
birth, the ability to produce testosterone may be normal, but it becomes progressively impaired later in adolescence as testicular fibrosis becomes more evident. Internal ducts follow ipsilateral gonadal histology. Tumors may arise, most commonly gonadoblastoma and dysgerminoma; however, in patients diagnosed and managed early, malignant degeneration is rare.

The diagnosis of true hermaphroditism is via chromosomal analysis, imaging, and hormonal studies which usually precede open or laparoscopic exploration. Imaging with sonography may reveal uterine and ovarian structures. Genitogram may outline a vagina, cervix, uterine cavity, and fallopian tubes. Endocrine studies include a HCG stimulation test in which a rise in serum testosterone following HCG administration is indicative of functioning testicular tissue. Histologic confirmation of the condition rests with gonadal biopsy.

1.3.4 Pure Gonadal Dysgenesis

Pure gonadal dysgenesis refers to a primary defect in gonadal formation. It may occur in patients with both 46,XX and 46,XY karyotype. Females with 46,XX will have a normal stature, sexual infantilism, and bilateral streak gonads. They will often present with delayed puberty and amenorrhea, and will be sterile but physically responsive to estrogen replacement. A 46,XX karyotype is not associated with gonadal neoplasia.

The 46,XO karyotype is found in patients with Turner's syndrome. It is a frequent malformation found in approximately 1 out of every 2,500 live female births. The physical findings of bilateral streak gonads, short stature, webbed neck, facial
dystrophism, and sexual infantilism are due to the total or partial loss of the chromosomal information from one of the two X chromosomes. Renal anomalies are common with horseshoe kidney being the most common. Cardiovascular anomalies found with this syndrome include bicuspid mitral valve and aortic coarctation. Short stature may respond to human growth hormone. Fertility is rare but reported.

Patients with pure gonadal dysplasia and a 46,XY karyotype will display variable degrees of undermasculinization dependent upon the extent of testicular dysplasia. Because testicular secretion of MIS may also be deficient, retained Müllerian ductal structures may be seen. Patients with 46,XY karyotype are at an increased risk of gonadoblastoma.

As a general rule, patients found early are reared as females with gonadectomy, clitoral reduction, vaginoplasty, and estrogen/prog-esterone replacement starting at puberty. In patients who present late and are committed to male gender assignment, they can undergo hypospadias repair, removal of Müllerian duct structures, and gonadectomy. They should receive androgen therapy starting at puberty.

1.3.5 Mixed Gonadal Dysgenesis

Patients with mixed gonadal dysgenesis have been categorized as having "partial gonadal dysgenesis" secondary to Y-chromosome mosaicism (Kim et al., 2002). Majority of the patients will have a 45,X0/46,XY karyotype, and it is characterized by a unilateral testis, a contralateral streak gonad, persistent müllerian ductal structures ipsilateral with the streak gonad, and varying levels of external genitalia undervirilization (Diamond, 2002). In the newborn, mixed gonadal dysgenesis is the second most common cause of
ambiguous genitalia, after congenital adrenal hyperplasia. There is an estimated incidence of gonadal tumor (gonadoblastoma or dysgerminoma) in approximately 15% to 20% of the individuals. Most commonly, this occurs in the dysgenic testis. These patients also have an increased incidence of Wilms' tumor and Denys-Drash syndrome characterized by progressive nephropathy (Diamond, 2002). Two-thirds of patients are reared as females. The individual may be raised as male if the patient is highly masculinized.

1.3.6 46, XX males without SRY gene

An increasing number of reports suggest that the male phenotype may develop even in the absence of SRY gene (Rajender et al., 2006). XX maleness is a rare syndrome with a frequency of 1 in 20,000 to 25,000 (de la Chapelle, 1981). Till date, many cases of XX males with or without SRY and apparently with no other Y-chromosome sequences have been reported (Zenteno et al., 1997; Abusheikha et al., 2001; Valetto et al., 2005). The cases with SRY gene arise because of an illegitimate recombination in the father placing the SRY gene on one of the X-chromosomes. These individuals develop ambiguous genitalia, undermasculinized male genitals or normal male genitals with complete or incomplete masculinization. These patients are infertile because of the absence of Y-chromosome.

1.4 Objectives of the present study

The studies of the above mentioned phenotypes have helped in the identification of a large number of genes, which are known to participate in the process of sex-determination. The mutations in the sex-determining genes have helped to explain the
ambiguities in the above cases, and to understand the genetic basis of gonad differentiation. Therefore, the objectives of the present study were to;

a) Understand the role of AR gene in sex reversals with androgen insensitivity syndrome among Indian patients.

b) Investigate the structural-functional correlations of the mutations in AR gene.

c) Investigate the relative levels of testosterone, LH and FSH in AIS, and their usefulness as indicators of androgen insensitivity.

d) Understand the role of SRD5A2 gene polymorphisms as genetic background in varying manifestation of androgen insensitivity, and to search for additional novel genetic components contributing to the phenotypic variation.

e) Understand the genetic basis of sex reversals represented by gonadal dysgenesis by analysing known sex-determining gene(s).

f) Search for additional chromosomal region(s) linked with gonadal dysgenesis in the cases unexplained by mutations in the known sex-determining genes, followed by in silico characterization of the region of interest.

g) Analyze unique cases of genital ambiguities to understand the etiology of sex reversals.

h) Offer genetic counseling to the affected families in order to prevent transmission of the defective AR alleles to the next generations.

i) Synchronize the results of the above studies and the evidences from previous studies to determine the hierarchy of all the known genes in sex-determination pathway.