APŽVALGINIS STRAIPSNIS

Human male sex determination and sexual differentiation: pathways, molecular interactions and genetic disorders

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Summary. *The complex mechanisms are responsible for male sex determination and differentiation. The steps of formation of the testes are dependent on a series of Y-linked, X-linked and autosomal genes actions and interactions. After formation of testes the gonads secrete hormones, which are essential for the formation of the male genitalia. Hormones are transcription regulators, which function by specific receptors. Ambiguous genitalia are result of disruption of genetic interaction. This review describes the mechanisms, which lead to differentiation of male sex and ways by which the determination and differentiation may be interrupted by naturally occurring mutations, causing different syndromes and diseases.*

Introduction

The male genetic sex is determined by the chromosomal set, which is usually 46,XY. This chromosomal pattern leads to a cascade of genetic events, which ends in the development of the male gonads and the secondary sexual characteristics. Steroids and peptide hormones, which are secreted by the gonads, are essential for the development of the internal and external genitalia. Hormone action mediates the phenotypic sex. The term sexual determination is used for the developmental processes leading to global testicular function. Sexual differentiation describes the specific hormone actions leading to the sexual phenotype of an individual. The gender of an individual is the sex of assignment and depends on normal sexual determination and differentiation (1). In this report we will focus on the most important and the most frequent cases of gonadal dysgenesis, disorders of androgen metabolism, and defects in androgen action with or without change in the degree of virilization.

Sex determination

The development of the gonadal, adrenal and urogenital systems is closely linked. Several genes are known to be involved in this process leading to the formation of the undifferentiated gonad (Fig. 1, with modification (1)). Among these genes two of them are the most important for the proper development of the bipotential gonad: nuclear receptor subfamily type 5, group A, member 1 gene (*NR5A1)* and Wilms' tumor suppressor gene (*WT1)*. *WT1* is a 10 exons gene and maps to chromosome band 11p13 (2). On the protein level, *WT1* shows 24 protein isoforms due to combinatorial splicing, alternative translation-initiation sites and RNA editing. The protein is composed of four zinc-finger domains, which are characteristic features of some transcription factors. *WT1* represents a transcription factor with tumor suppressor activity. It is expressed predominantly in the embryonic kidneys and gonads. The WT1 protein mediates the mesenchymal-epithelial transition and differentiation during morphogenesis of the kidneys and gonads by repressing genes that encode cell proliferation factors and by activating genes that encode markers of epithelial cell differentiation. There are two alternative splicing sites of intron 5 and intron 9 in *WT1* gene. Splicing of intron 9 has a great biological importance and results in the inclusion or exclusion of 3 amino acids: lysine, threonine and serine (KTS respectively), yielding the KTS+ isoform, when the amino acids are included and the KTS– isoform, when excluded. The precise ratio of the KTS+/KTS– isoforms is crucial for the normal function of the *WT1* gene. The KTS-negative isoform of the WT1 protein associates and achieves synergy with steroidogenic factor 1 (SF1). SF1, in turn, promotes expression of

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the gene-encoding Müllerian inhibiting substance (MIS). The degree to which the synergy between WT1 and SF1 is interrupted determines the severity of gonadal abnormalities in 46,XY individuals. In contrast, in 46,XX individuals, an intact *WT1* gene has not been shown absolutely necessary for normal female development; these patients have less severe or no gonadal abnormalities, however, they still show renal symptoms as well. The loss of the more abundant KTS+ isoform leads to Frasier syndrome, which in Online Mendelian Inheritance in Man (OMIM) is numbered by #136680 (3, 4). Frasier syndrome is characterized by nephropathy, male pseudohermaphroditism and complete gonadal dysgenesis. Constitutional missense mutations in the Wilms' tumor suppressor gene are usually associated with Denys-Drash syndrome (OMIM #194080), which is described by male pseudohermaphroditism, nephropathy and Wilms' tumor (5). Wilms' tumor (nephroblastoma) is the most frequent tumor of the kidney during infancy. It is an embryonic malignancy and to date, only one Wilms' tumor candidate gene, *WT1*, which can be a cause of this malignancy, has been identified. WAGR (Wilms' tumor, aniridia, genital abnormality, mental retardation) is another syndrome, which is caused by deletion of *WT1* and other several closely linked genes and presents a typical contiguous gene syndrome (OMIM #194072).

Steroidogenic factor 1 is the product of the *NR5A1* gene. This gene is composed of 7 exons, lies on human chromosome 9q33.3 and encodes an orphan nuclear receptor molecule, which can bind to promoter elements of the steroid hydroxylases (6). The mRNA of this gene is expressed in the urogenital ridge, which forms gonads and adrenals. SF1 promotes anti-Müllerian hormone (AMH) expression by binding to upstream regulatory elements of the *AMH* gene (7). Both, SF1 and the zinc-finger transcription factor GATA-4 synergistically activate the *AMH* promoter. Another co-factor for AMH regulation is the SRY-related SOX8, which acts through a protein-protein interaction with SF1. SF1 also activates the receptor of this Sertoli cell specific hormone, by binding to MIS II receptor MISRII. Mutations in SF1 results in primary adrenal insufficiency and male pseudohermaphroditism in 46,XY individuals (OMIM #184757) (8). In individuals with a 46,XX karyotype, the development of the ovary is not affected by mutations in SF1 (9). During the first week of life patients present with adrenal failure.

It has been proven, that the testis-determining factor is on chromosome Yp11.31. The gene was termed *SRY* (sex determining region on the Y chromosome) (10). This is a one-exon gene, with the highly conserved 79-amino acid high-mobility group (HMG) box sequence. This gene triggers testes formation from the indifferent gonads. Mutations in *SRY* cause pure gonadal dysgenesis with XY male-to-female sex reversal (Swyer syndrome) (OMIM #306100) or true hermaphroditism (OMIM #235600) (11). Pure gonadal dysgenesis is restricted to those cases with gonadal dysgenesis including streak gonads, normal Müllerian structures and normal female external genitalia. The patients are of normal stature and have no signs of Turner syndrome. Lack of the secondary sexual characteristics is common. True hermaphroditism is characterized by the presence of both ovarian and testicular tissue in the same individual.

Male sex determination in the absence of the Y-chromosome and another SRY-related HMG box-containing gene, results in XX sex-reversed individuals (12). These patients can be categorized into

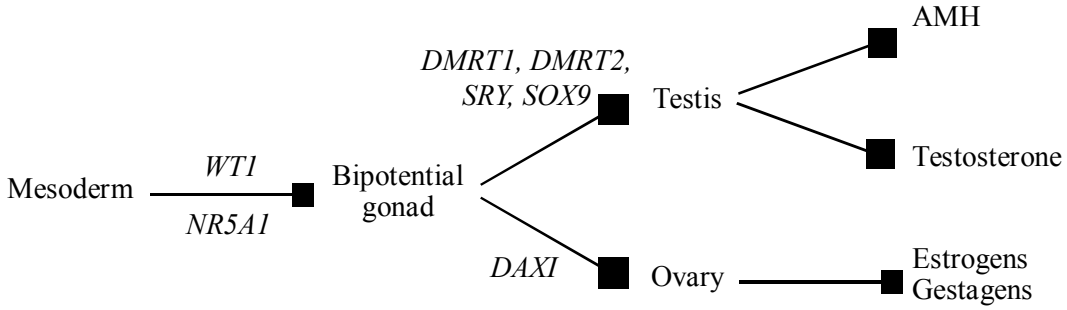

Fig. 1. **Genes involved in sex determination and subsequent gonadal differentiation**
There are several genes involved in the formation of the bipotential gonad and later, testes and ovary. This image is a coarse summary of gene interactions, which are essential for sex determination, gonad differentiation and subsequent steps of sexual differentiation. The function and role of these genes are mainly known from cases with disturbances of sex determination (sex reversal) and from animal models of mammalian sex determination.

two main groups: SRY-positive XX males and SRY-negative XX males. 46,XX maleness is a rare disorder that occurs in about 1 per 20,000 males (13). Male normal or ambiguous external genitalia, two testes and azoospermia characterize all patients. Müllerian structures are absent. About 80% patients have the *SRY* gene. This is the result of *de novo* abnormal crossover between the Y chromosome and the X chromosome, resulting in presence of the *SRY* gene on the X chromosome. *SRY* and *SOX9* (14) are genes known to be associated with XX male syndrome. At least one more unknown gene is implicated.

SRY is just one member of a family of genes having in common the HMG box. These genes have come to be known as *SOX* (*SRY*-related HMG box) genes. *SOX9,* another SRY-related HMG-box containing gene, is a typical transcription factor with discrete DNA binding and transcriptional trans-activation domains. *SOX9* is composed of the HMG domain, a proline, glutamine and alanine (PQA) domain and a proline, glutamine and serine-rich domain (PQS). The gene has 3 exons and it maps to chromosome 17q24.3-q25.1. The HMG box region of *SOX9* binds to the sequences AACAAT and AACAAAG, typical of *SRY* and other SOX proteins and, in addition, binds to the variant sequences ATGAAT and CACAAT, found in the chondrocyte-specific enhancer in the first intron of the human type II collagen gene *COL2A1*. This gene is expressed during Sertoli cell differentiation and it is essential for formation of the extra cellular matrix of cartilage, too. It was concluded from its expression profile that *SOX9* acts downstream of *SRY* and may be regulated by *SRY*. *SOX9* mutations cause both autosomal male-to-female sex reversal with gonad dysgenesis and camptomelic dysplasia (OMIM #114290), which is characterized by defects of the chest, ribs and sternum and bowing of the long bones (15, 16).

Another gene involved in adrenal, ovarian and testicular development is the *DAX1* gene. *DAX1* (DSS, AHC, X-linked; gene 1) is part of the dosage sensitive sex reversal locus-adrenal hypoplasia congenital-critical region on the X chromosome. Its official gene symbol is *NROB1* (nuclear receptor subfamily O, group B, member 1). *DAX1* is composed of a C-terminal ligand-binding domain (LBD) and a putative DNA-binding (DBD) domain. The *DAX1* gene has two exons. This gene is expressed in human ovarian tissue, in the ventromedial nucleus of the hypothalamus, gonadotropic cells of the pituitary gland and in the adrenal cortex. Expression during ovarian development, but not testicular formation, implies its important role for the ovarian formation. Duplication of the DSS region with the *DAX1* gene results in XY gonadal dysgenesis with male-to-female sex reversal (OMIM #300018) (17). The mutations within the gene are responsible for adrenal hypoplasia congenital (AHC) and hypogonadotropic hypogonadism (OMIM #300200) (18).

Deletions of chromosome 9p region have been associated with sex reversal in 46,XY individuals. Two genes named *DMRT1* and *DMRT2* were identified on chromosome 9p24.3 (19). *DMRT1* in 9p24.3 is composed of 5 exons. The flanking *DMRT2* gene consists of 3 exons. *DMRT1* is expressed in male genital ridge and no transcripts were found in the female gonad. Deletions of these genes have been detected in patients with 46,XY male-to-female sex reversal (19).

Thus, defects in developmental genes responsible for gonadal differentiation lead to a global complete or partial gonadal dysgenesis. However, in the majority of cases with gonadal dysgenesis in 46,XY patients, no genetic defect can be distinguished until today. The characterization of unknown genes in the near future, which affect the default pathways of sex determination and gonad differentiation will be very important to the diagnosis, treatment and counseling of patients with a sexual determination disorder (1).

Principles and disorders of sexual differentiation

Although the chromosomal basis of sex is determined at the moment of conception, the internal structures are indifferent until 6 weeks gestation. These structures consist of gonads, primordial germ cells and Wolffian (mesonephric) and Müllerian (paramesonephric) ducts. In males, the Wolffian ducts become the male internal genitalia, which consist of the epididymis, the vas deferens, and seminal vesicles. Internally, the process results in the ejaculatory duct system for sperm and is complete by 14 weeks gestation. Similar to the internal structures, the external structures are initially indifferent and consist of the urogenital tubercle, the urogenital swellings, and the urogenital folds. Male external genitalia are complete by 14 weeks gestation. The urogenital tubercle will become the glans penis, the urogenital swellings fuse into the scrotum and the urogenital folds become the shaft of the penis. In the absence of a testis, the Müllerian duct differentiates into fallopian tube, uterus and the upper portion of the vagina. Externally genital tubercle, urogenital folds and urogenital swellings become clitoris, labia minora and labia majora, respectively.

The presence of ovaries is not required for either internal or external female genital formation.

The epithelium of the Müllerian duct is a tissue with inducer capability for the Müllerian ducts derivatives. Gene targeting of *LHX1* (homologue *LIM1*) in female mice leads to absence of uterus and fallopian tubes, making an essential factor of development of female internal genitalia (20). Müllerian duct agenesis may be the result of *EMX2* mutations that (at least in mice) lead to renal anomalies and intrauterine death (21). In humans, aberrant expression levels of EMX2 have been described in patients with endometriosis (22). The testes consist of two main types of the cells: Sertoli and Leydig cells. Anti-Müllerian hormone, secreted by Sertoli cells, is important for suppression of Müllerian ducts. To exert the action of AMH, high concentrations of this hormone and active binding to a membrane receptor in the mesenchymal cells, surrounding the Müllerian ducts are necessary. The anti-Müllerian gene expression is regulated by SF1, which binds to the AMH-gene promoter and activates its expression. The lack of anti-Müllerian hormone and insensitivity to this hormone has been described in "persistent Müllerian duct syndrome" (PMDS) (OMIM #261550). This syndrome is characterized in 46,XY males by the presence of fallopian tubes and uterus. The external genitalia are male, as steroid formation is normal. PMDS, due to both, AMH and AMH-type receptor gene mutations, is inherited in an autosomal recessive fashion (1).

Testosterone synthesis in the Leydig cells of the developing testes is controlled during early fetal life by human chorionic gonadotrophin (hCG) and later by luteinizing hormone (LH) itself. These two hormones stimulate testosterone synthesis in Leydig cells via the luteinizing hormone receptor (LHR). The LHR gene is localized on chromosome 2p21, and spans over 90 kb, has a coding region divided into 11 exons (1). Unresponsiveness to LH leads to Leydig cell agenesis and thus to defective sexual differentiation. The result is a completely female phenotype, but sometimes-incomplete virilization happens, especially during puberty when testosterone levels raise, due to partial receptor responsiveness (23).

The pathway of androgen synthesis

Some steps in the pathway of steroid synthesis are shared between glucocorticoids, mineralocorticoids and sex steroids, like testosterone or estrogen (estradiol). The defects within steroid synthesis will affect all or at least two of the final metabolites in the testes and the adrenals (24) (Fig. 2, with modification (1)). Steroid hormones are synthesized within mitochondria and the endoplasmic reticulum. The steroidogenic acute regulatory protein (StAR) mediates acute stimulation of steroid synthesis (25). Mutations in the *StAR* gene lead to severe lack of adrenal steroidogenesis and lack of virilization in 46,XY individuals (OMIM #201710). It was concluded that the congenital lipoid adrenal hyperplasia phenotype is the result of two separate events: the primary defect is genetic loss of steroidogenesis that is dependent on StAR protein; there is a subsequent loss of steroidogenesis that is independent of StAR due to cellular damage from accumulated cholesterol esters. The adrenal cortex becomes engorged with cholesterol and cholesterol esters; the resulting deficiency in adrenal steroidogenesis leads to salt wasting, hyponatremia, hypovolemia, hyperkalemia, acidosis, and death in infancy, although patients can survive to adulthood with appropriate mineralocorticoid- and glucocorticoid-replacement therapy (26). Intra-uterine survival of affected children is possible because placental steroidogenesis is not StAR-dependent. *StAR* mutations and all other defects of androgen biosynthesis are inherited in autosomal recessive fashion and both genders are affected.

The enzyme 17-alpha-hydroxylase is an enzyme that mediates 17-alpha-hydroxylase and 17,20-lyase activities; it catalyses both, 17-alpha-hydroxylation of pregnenolone and progesterone and 17,20-lysis of 17-alpha-hydroxypregnenolone and 17-alpha-hydroxyprogesterone. The gene *CYP17C17*, which encodes this enzyme, is the sole member of a unique gene family within the P450 super gene family. The adrenal P450c17 gene lies on chromosome 10q24-q25. Patients with 17-alpha-hydroxylase and 17,20-lyase enzymes deficiency (OMIM #202110) have hypertension, because of excessive production of corticosterone and deoxycorticosterone, males are undervirilized at birth and females have primary amenorrhea and lack of secondary sexual characteristics. Another important enzyme of steroid genesis is 3β-hydroxysteroid dehydrogenase (3β-HSD). There are known two isoenzymes in humans. Both genes are located on chromosome 1p13.1 and consist of four exons. The gene for the type I enzyme is expressed mainly in the placenta and peripheral tissues. No mutations have been reported yet. However, in the type II gene (OMIM #201810), which is expressed predominantly in the adrenals and gonads, nonsense mutations and a frameshift mutation ware identified. These mutations give rise to congenital adrenal hyperplasia manifested by salt-wasting and incomplete masculinization in males, e.g. hypospadias and gynecomastia.

The functional gene for adrenal 21-hydroxylase, *CYP21A2,* is located about 30 kb from a non-func-

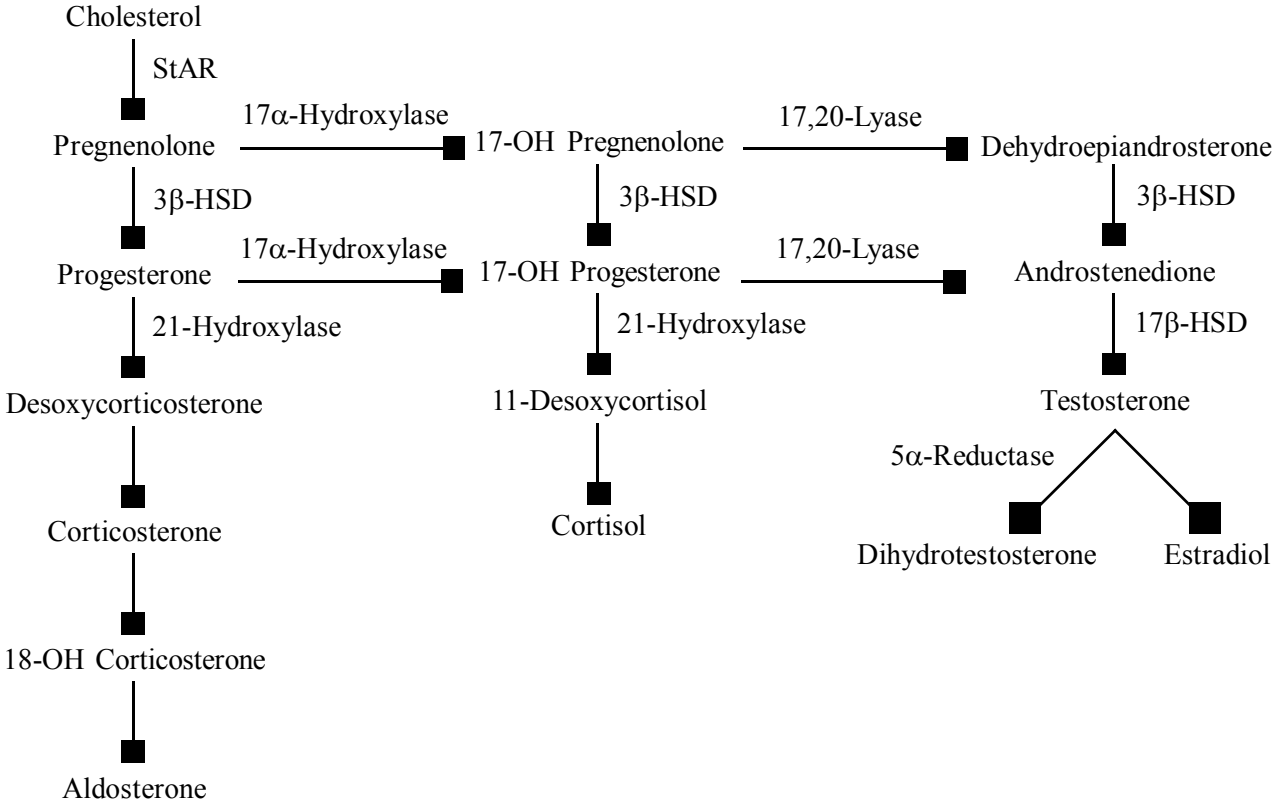

Fig. 2. **Pathways and metabolites of androgen biosynthesis**

The androgens, mineralocorticoids, glucocorticoids are synthesized in adrenal glands. The figure shows metabolites and enzymes, which were mentioned in the text and are important for the development of normal sexual development. Mutations in the genes for these enzymes may lead to ambiguous genitalia or salt-wasting syndromes. Depending on the karyotype and the phenotype of the individual, male or female pseudohermaphroditism happens.

tional pseudogene, *CYP21A2P*, on chromosome 6p in the human leukocyte antigen (HLA) gene cluster. *CYP21A2* and *CYP21A2P* consist of 10 exons. The gene has five normal variants (insL9, K102R, D183E, S268T, and N493S). This gene encodes a protein, which is at most 28% homologous to other cytochrome P450 enzymes that have been studied. 21-hydroxylase deficiency (OMIM #201910) is the most common cause of congenital adrenal hyperplasia disorder involving impaired synthesis of cortisol and often aldosterone. A classic form with severe enzyme deficiency and prenatal onset is distinguished from a non-classic form with moderate enzyme deficiency and postnatal onset. The classic form is further divided into the simple virilizing form (~25% of patients) and the salt-wasting form in which aldosterone production is inadequate (>75% of patients).

Later steps in androgen synthesis do not affect glucocorticoid and mineralocorticoid formation. 17β-hydroxysteroid dehydrogenase (17β-HSD) converts androstenedione to testosterone within testes and is expressed only in the testes, compatible with it is important role in testicular androgen formation. There are at least five different isoenzymes of 17β-HSD. An only mutation in the type 3 enzyme is responsible for defective sex differentiation. The 17β-HSD 3-gene has locus on chromosome 9p22 and spans 11 exons. Mutations in this gene cause a severe virilization defect in 46,XY individuals, however, during puberty strong signs of virilization were shown (OMIM #264300) (27), which causes the question for surgical removal of the gonads during infancy just to avoid a crisis of gender identity during puberty.

5α-reductase catalyses the conversion of testosterone to dihydrotestosterone (DHT) in the peripheral target cells. The two isoenzymes of 5α-reductase are expressed in diverse tissues. The type 2 enzyme plays a specific role in male sexual differentiation. The gene is localized on chromosome 2p23 and consists of 5 exons. In 5α-reductase deficiency, DHT formation is severely diminished (28). However, testosterone level is normal or elevated. Affected individuals with 46,XY karyotype usually born with ambiguous genitalia, but phenotype may be highly variable (29, 30). The diffe-

rentiation of Wolffian structures is normal. The disease is inherited in an autosomal recessive fashion (OMIM #184753).

Molecular mechanism of androgen action

Androgen action is dependent on normal expression of a functionally intact androgen receptor (AR). The AR is a hormone-activated DNA-binding transcription factor of androgen regulated target genes. This gene has been mapped to the X chromosome. The androgen receptor gene is more than 90 kb long and codes for a protein that has 3 major functional domains (31–33). The N-terminal domain, which serves a modulator function, is encoded by exon 1. The DNA-binding domain is encoded by exons 2 and 3. The androgen-binding domain is encoded by 5 exons (34). Upon entering into target cells, androgens bind specifically to the inactive AR, which is usually located within the cytoplasm (35). The binding results in dissociation of the different proteins from the AR that are initially associated with steroid receptors in a heteromeric complex (e. g. heat shock proteins) and promotes the activation and nuclear translocation of the AR. An important further step in the transactivation cascade prior to receptor binding to target DNA consists in homodimerization of two AR proteins. This androgen-dependent process is mediated by distinct sequences within the second zinc finger of the DNA-binding domain as well as through specific structural N-C-terminal interactions. The AR homodimer binds to hormone responsive elements (HRE) within the promoter region of the androgen-regulated target genes. This results in up- and downregulation, eliciting specific biological effects.

Mutations in this gene cause androgen insensitivity syndrome (AIS) (OMIM #300068). The most commonly observed molecular alterations of the AR-gene are missense mutations, predicting for an isolated amino acid exchange. Depending on the localization of the amino acid exchange within the androgen receptor, various molecular mechanisms, altering AR activity have been elucidated. Mutations within the ligand-binding domain may alter androgen-binding capacity; mutations within the DNA-binding domain affect receptor binding to target DNA. Mutations can impair androgen receptor mRNA stability and lead to additional dysfunction of androgen action. Due to the X-chromosomal recessive inheritance, only genetic male individuals (46,XY) are affected by AIS, while female carriers may be conductors. The clinical symptoms in AIS result from the combination of the defective androgen action in androgen-dependent target tissues. The syndrome is characterized by the feminization (undermasculinization) of the external genitalia at birth, normal testes, absent or rudimentary Müllerian structures, normal or elevated synthesis of testosterone and normal conversion to DHT, normal or increased LH production by the pituitary gland, deficient or defective androgen binding capacity in genital skin fibroblast. Androgen insensitivity syndrome can be subdivided into three phenotypes: complete androgen insensitivity syndrome (CAIS), partial androgen insensitivity syndrome (PAIS) and mild androgen insensitivity syndrome (MAIS). The patients with complete androgen insensitivity syndrome acquire a normal female body shape and breast development; they have female external genitalia with short and blind-ending vagina and normal testes. The pubic and auxiliary hair is scanty. The partial androgen insensitivity syndrome can be subdivided into few forms. The predominantly female ('incomplete virilization') form consists of inguinal or labial testes, clitoromegaly and labial fusion, distinct urethral and vaginal openings or a urogenital sinus. The ambiguous form is characterized by micro phallus (<1 cm), descended or undescended testes, perineoscrotal hypospadias or urogenital sinus, and gynecomastia in puberty. The predominantly male form is characterized by simple (glandular or penile) or severe (perineal) "isolated" hypospadias with a normal-sized penis and descended testes or severe hypospadias with micropenis, bifid scrotum, and either descended or undescended testes. The mild androgen insensitivity syndrome form has symptoms, like impaired spermatogenesis and/or impaired pubertal virilization with gynecomastia in puberty.

Conclusion

Sex determination and subsequent sexual differentiation is a complex process of gene interactions, metabolic pathways of steroid synthesis and gene activation as well as hormone regulation by the interaction of ligands with their corresponding receptors. Each individual step, which is mediated by gene products, both as protein factors or enzyme, is prone to phenotypic change due to mutations in the corresponding gene. We have shown that all modes of inheritance are possible: Y-chromosomal (SRY), X-chromosomal (DAX1/AR), autosomal recessive (e.g. 5α-reductase) and autosomal dominant (the rest). The vast majority of cases are not familial, because the affected individual cannot reproduce due to sterility. There are a lot of diseases, which can cause ambiguous genitalia. This review aims to describe the most important mechanisms and genes, which play a role in male sex determination and differentiation and syndromes as well as diseases, caused naturally occurring mutations.

Vyriškosios lyties determinacija ir diferenciacija: molekuliniai mechanizmai ir ligos

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Raktažodžiai: vyriškosios lyties determinacija, lyties diferenciacija, interseksualiosios būklės.

Santrauka. Vyriškosios lyties diferenciacija yra sudėtingas procesas. Bipotencinės gonados, o vėliau sėklidžių vystymasis, yra susijęs su lytinių chromosomų ir autosomų genų bei jų produktų sąveika. Sėklidės išskiria steroidinius hormonus ir peptidus, kurie yra būtini normaliam išorinių ir vidinių lytinių organų formavimuisi. Hormonai veikia per specifinius receptorius, funkcionuojančius kaip transkripcijos reguliatoriai. Šių mechanizmų pažeidimai lemia interseksualiąsias būkles. Šiame straipsnyje aprašomi vyriškosios lyties vystymosi mechanizmai ir sutrikimai, kurių atsiranda dėl lyties vystymąsi veikiančių genų mutacijų.

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