CLINICAL AND MOLECULAR CHARACTERISTICS OF PATIENTS WITH 46,XY DSD DUE TO NR5A1 GENE MUTATIONS

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Steroidogenic factor 1 (SF1, NR5A1) is a nuclear receptor that regulates multiple genes involved in adrenal and gonadal development, steroidogenesis, and the reproductive axis. Human mutations in SF1 were initially found in patients with severe gonadal dysgenesis and primary adrenal failure. However, more recent case reports have suggested that heterozygous mutations in SF1 may also be found in patients with 46,XY partial gonadal dysgenesis and underandrogenization but normal adrenal function. We have analyzed the gene encoding SF1 (*NR5A1*) in a cohort of 310 Russian patients with 46,XY disorders of sex development (DSD). Heterozygous SF1 variants were found in 36 out of 310 (11.6%) of cases, among them 22 were not previously described. We have not found any phenotype-genotype correlations and any clinical and laboratory markers that would allow to suspect this type of before conducting molecular genetic analysis.

KEYWORDS: SF1; steroidogenic factor 1; gonadal dysgenesis; disorders of sex development; hypospadias; NR5A1.

КЛИНИЧЕСКИЕ И МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИЕ ХАРАКТЕРИСТИКИ ПАЦИЕНТОВ С НАРУШЕНИЕМ ФОРМИРОВАНИЯ ПОЛА 46,XY, ОБУСЛОВЛЕННЫМ МУТАЦИЯМИ В ГЕНЕ NR5A1

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Орфанный ядерный рецептор — стероидогенный фактор 1, кодируемый геном *NR5A1,* является одним из ключевых регуляторов стероидогенеза, формирования стероидных тканей и их регуляции гипоталамо-гипофизарной осью. Изначально считалось, что мутации в гене *NR5A1* характеризуются сочетанной надпочечниковой и гонадной недостаточностью, но данный фенотип оказался редким проявлением дефекта NR5A1. В то же время нарушения функции NR5A1 — одна из частых причин изолированных форм нарушения формирования пола (НФП) 46,XY. Мы проанализировали распространенность мутаций в гене *NR5A1* среди 310 российских пациентов с НФП 46,XY и выявили изменения в этом гене в 36 случаев (11,6%). Среди обнаруженных мутаций 22 ранее описаны не были. Нами не установлено наличие фенотип-генотипической корреляции даже в пределах одной семьи. Проведенная работа не выявила каких-либо клинических и гормональных маркеров, которые позволили бы заподозрить данный нозологический вариант НФП 46,XY до проведения молекулярно-генетического анализа. Для более объективных выводов необходимо продолжить наблюдение за данной группой пациентов, особенно при достижении ими периода пубертата.

КЛЮЧЕВЫЕ СЛОВА: нарушение формирования пола, гипоспадия, стероидогенный фактор, NR5A1, дисгенезия гонад.

RELEVANCE

Disorder of sex development (DSD) is a condition caused by a clinical and biochemical manifestation of discordance between the chromosome, the gonad and/or the phenotypic sex [1]. Classical 46,XY DSD variants occur with approximately 1/20,000 frequency; however, if proximal forms of hypospadias and bilateral cryptorchidism were to be included into DSD variants, the frequency of this pathology would rise to 1/300–500 babies [2]. Given the clinical importance of a correct diagnosis and the psychological aspects related to the determination of the child's sex, molecular genetic methods are usually the preferred method of establishing the condition's aetiology, as they are considered the most precise and decisive in the treatment of patients with 46,XY DSD. However, although over 400 genes somehow involved in the sex differentiation process have been described, the percentage of 46,XY DSD cases in which the condition's genetic cause has been established amounts to 50%–60% only.

NR5A1 gene that codes NR5A1 protein is one of the key genes involved in sex development.

NR5A1 (Steroidogenic factor 1, SF1) is a regulatory protein and an orphan nuclear receptor [3].

Since the discovery of NR5A1 in 1992, its biological significance has remained somewhat obscure. Originally, the NR5A1 function was identified as regulation of steroidogenesis enzymes expression in steroidogenic tissues. However, it was subsequently found that this protein is instrumental for adrenal gland and gonad differentiation, as well as genesis of ventromedial hypothalamus nucleus and hypophysis gonadotrophs [4].

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The first clinical cases associated with *NR5A1* gene mutations were described in the studies of patients with 46,XY DSD and primary chronic adrenal insufficiency (PCAI) [5]; however, it was found that adrenal insufficiency cases were rare, and most of such patients had isolated disorders of gonad genesis and function while having no adrenal insufficiency [6].

As clinical cases amassed and genetic studies became more accessible, several international researchers proved that heterozygous mutations in *NR5A1* accounted for 10%–20% of all forms of 46,XY DSD without PCAI, *i.e.*, they are the second leading cause of 46,XY DSD, whereas variants in the androgenic receptor gene are the top one [6, 7, 8].

Phenotypical manifestations of 46,XY DSD in patients with *NR5A1* defects show a significant variety, from almost female external genitalia showing slight clitoromegaly and absence of palpable gonads to scrotal hypospadias and cryptorchidism. Müllerian duct derivatives as gonad dysgenesis markers may be identified at different stages of development, but they are not obligatory. Moreover, even though this disease is classified within the category of gonad dysgenesis, some patients registered as male show a spontaneous masculinisation at puberty [9, 10, 11], and that masculinisation does not always correlate with the extent of atypical genitalia at birth.

All of the above emphasises the complexity of diagnosing and treating patients with this 46,XY DSD variant.

CASE

In this study, we summarised clinical and laboratory characteristics of 36 Russian patients with 46,XY DSD and evidence of mutations of *NR5A1* gene.

Informed consent was obtained from all examined patients; for those under 15, informed consent forms were signed by their legal representatives as per the research protocol.

MATERIALS AND METHODS

This study examined DSD patients with 46,XY karyotype. DSD was defined as discordance between the chromosome and the phenotypic sex. The Prader scale was used for evaluation of external genitalia dysmorphology. Patients having 46,XY DSD and syndromic pathology were excluded from this study.

A comprehensive examination was conducted, including: Prader scale-based evaluation of external genitalia morphology; ultrasonic examination (US) of pelvis, inguinal canal and scrotum; examination of hormonal status: luteinising hormone (LH), follicle-stimulating hormone (FSH), testosterone and estradiol (E2). In some of the patients, the level of anti-Müllerian hormone (AMH) was examined and chorionic gonadotropin tests were performed.

Molecular genetic analyses were performed in the laboratory of Hereditary Endocrinopathy Department, Russian National Medical Research Centre for Endocrinology. The gDNA was isolated from peripheral white blood cells through a standard method (Pure Link, Genomic DNA Mini Kit, Life Technologies, USA). NGS method was used. A primer panel developed in Hereditary Endocrinopathy Department, Russian National Medical Research Centre for Endocrinology was used in multiplex PCR tests and Ion Ampliseq™ Custom DNA Panel (Life Technologies, USA) sequencing. The Disorder of Sex Development primer panel covers the following genes' coding areas: *AKR1C2, AKR1C4, AMH, AMHR2, AR, ARX, ATRX, CBX2, CYB5A, CYP11A1, CYP17A1, DHCR7, DHH, EMX2, ESR2, FGD1, FGF9, FGFR2, FKBP4, FOXF2, FOXL2, HOXA13, HSD17B3, HSD3B2, ICK, LHCGR, LHX1, LHX9, MAMLD1, MAP3K1, MID1, NR0B1, NR5A1, POR, PTGDS, SOX9, SRD5A2, SRY, STAR, SUPT3H, TSPYL1, WNT4, WT1, ZFPM2*.

Preparation of libraries was carried out as per the manufacturer guidelines. The sequencing was performed with PGM (Ion Torrent, Thermo Scientific, USA) or Illumina MiSeq (Illumina, USA) semiconductor sequencer. The processing of the sequencing results was carried out with Torrent Suite 4.2.1 (Ion Torrent, Life Technologies, USA) or Genome Analysis ToolKit (GATK) ver. 4.1.2.0 (Broad Institute, Cambridge, MA, USA) software. Annotation of nucleotide sequence variants was performed with ANNOVAR ver. 2018Apr16 software package. Following the analysis of data, obtained mutations were confirmed using Genetic Analyzer Model 3130 (Life Technologies, USA) sequencer. Assessment of nucleotide sequence variants' pathogenicity was carried out as per international and Russian guidelines [12, 13]. The *NR5A1* coding sequence numbering is based on GenBank reference DNA sequence NM_004959.3 (http://www.ncbi.nlm.nih.gov/genbank).

FINDINGS

The study included 310 patients with 46,XY DSD examined in 2015–2019. Molecular genetic tests identified heterozygous nucleotide variants in *NR5A1* in 36 patients, which is 11.6% of the total number of 46,XY DSD patients. Among these 36 patients, there were two families with two sibs having 46,XY DSD. None of the 46,XY DSD patients had any biallelic mutations in the *NR5A1* gene.

Out of these 36 patients, 19 were registered as females and 17 as males at birth. Out of those registered as females, four had female external genitalia, and no doubts had existed as to their sex determination. A further seven had moderate clitoromegaly (length up to 1.5 cm) and unexpressed labia majora folds with palpable gonads on one or both sides (Prader 2 masculinisation). A further seven had more expressed clitoromegaly (length up to 2.5 cm), glans formation, narrow vagina entrance, and scrotum-looking labia majora with palpable gonads on one or both sides (Prader 3). A similar morphology of external genitalia was found in ten patients registered as males at birth. Six boys had scrotal hypospadias and a narrow opening at the base of penis (Prader 4). Prader 4 was also found in one child registered as female.

Two out of four female patients who had normal female external genitalia at birth were examined at the age of 2 and 4 in connection with inguinal hernia which were found to contain gonads. The other two first sought care at the age of 12 and 14 in connection with enlarged clitoris, low voice, development of male body hair and absence of breast development.

As the above data show, most of the patients had ambiguous external genitalia classified as "undetermined" (Prader 3). Interestingly, patients in this group had equally ambiguous external genitalia, yet their distribution by sex was almost even: eight females and ten males. In those registered

Table 1: Prader scale classification of external genitalia morphology

Sex registered at birth	Morphology of external genitalia			
	Female/ Prader 1	Prader 2	Prader 3	Prader 4
Female	4	7	7	1
Male	0	0	11	6

as females at birth, external genitalia irregularities observed included clitoromegaly (quite often with glans formation), a moderately developed cavernous body, a single urogenital sinus, and a high posterior labial commissure; quite often, palpable gonads were found in their scrotum-looking labia majora. In those registered as males, the phenotype was classified as perineal hypospadias, penis underdevelopment, and unilateral or bilateral cryptorchidism.

Out of those registered as males, five patients had reached puberty age at the time they were observed (age 13–18, Tanner stages 3–5). In all of them, spontaneous regular puberty and adequate development of secondary sex characteristics were observed (earlier, we described a case of familial NR5A1 deficiency with spontaneous puberty [11]). Hormonal tests found the levels of FSH at 8.0–14 u/l (median: 9.1; normal 0.7–11.1); LH at 5.32–8.1 u/l (median: 5.5; normal 1.3–9.6); testosterone varied between 11 nmol/L and 26.6 nmol/L and its level was inversely correlated with the patients' age. Table 2 presents the patients' clinical and hormonal characteristics.

32 children were examined before the age of 1 due to ambiguity of external genitalia. Out of that number, hormonal tests were performed in 12 children under 12 months; in eight of them, higher FSH was found: 5–14 u/l (median: 7.2; normal for children under 1: <3.3 u/l). However, their AMH was at or under 18 ng/ml, whereas the remaining four children had normal FSH levels but much higher AMH (40–60 ng/ml), which indicates different degrees of Sertoli cell dysfunction [14]. Out of the 19 patients registered as females, notwithstanding low AMH levels typical for that cohort, Müllerian duct derivatives (MDD) were found in four patients only. Among the patients registered as males, in three of them US detected an additional cord-type mass behind the urinary bladder. However, it is important to keep in mind that US of pelvis is not always conducted in patients registered as males, and quite low US sensitivity may not be sufficient to detect underdeveloped MDD.

In all 12 children who were examined in their first year, LH was found to be within the normal range (0.2–0.4 u/l), which indirectly indicates postnatal preservation of steroidogenesis in testes. Chorionic gonadotropin tests were performed in eight children under 1. Testosterone increase was between 4.6 nmol/L and 33 nmol/L, which also indicates a relative preservation of Leydig cells function (Table 2).

12 patients registered as females had gonads removed at varying ages. For five of these patients, findings of histopathological examinations of the removed gonads were available. In all of them, weak development of interstitial cells, patchy fibrous degeneration, Leydig cell agglutination, and various amounts of Sertoli cells were observed. Neither ovotesticular nor streak gonads were described in any of these cases.

The sex of one patient originally registered as female was changed to male. At birth, the child's external genitalia were closer to male (Prader 4) and testes were located in the lower third of the inguinal canal. On examination at the age of 6 weeks, normal levels of gonadotropins were detected; testosterone increase in a chorionic gonadotropin test was 15 nmol/L, suggesting preservation of gonad steroidogenesis. Given these findings and the parents' consent, sex change was advised.

Characteristics of *NR5A1* gene mutations

In 36 patients, 31 variants in *NR5A1* gene were found, including 15 not previously described.

The p.R313C mutation has been previously described; it was found in three non-related patients whose phenotypes varied substantially, from moderate clitoromegaly to a well-developed scrotum and scrotal hypospadias. One patient who was registered as female was first examined at the age of 12 due to progressing masculinisation. Two other patients with this mutation had Prader 3–4 and were registered as males. At the time of this study, they hadn't reached their puberty and the preservation of their testes' function could not be assessed. In one patient, a mutation in the same triplet was found (p.R313H); this mutation has been described already. The patient had atypical genitalia at birth (Prader 3) at birth, had a spontaneous puberty with adequate masculinisation and penis growth; the patient's gonadotropin level at the age of 15 was slightly over the upper limit of the normal range, and testosterone level was normal for the age, whereas the testes' volume was at the lower limit of normal for the age (12–12 ml).

A substantial phenotypical variability was also observed in three patients who had the p.R84H mutation (has been described earlier): two such patients who were registered as females had atypical genitalia at birth (Prader 3) with gonads palpable in the inguinal canals, and one patient who was registered as male had Prader 4 without cryptorchidism. Two sibs registered as males had different severity of external genitalia ambiguity; they had the same p.H317Q*fs*X17 mutation (already described). In both of them, spontaneous puberty was observed. However, at the age 16 and 18, both of them had gonadotropin levels slightly over the upper limit of the normal range (just as in the case described above), while their testosterone levels were low to normal and testes' size at the lower limit of normal (Table 2).

Table 2: The patients' phenotypical, hormonal and molecular genetic characteristics

	Sex as registered	Age	Prader phenotype	MDD	Gonad location	FSH/LH	T nmol/L / after a chorionic gonadotropin test	AMH (ng/ml)	Genetic data	Pathogenicity
1	F	4 m	3			10.6/2.2	1.3/7.59	12	c.del1273_1278	
2	F	11 m	3	no	none	14/	10.6	13.7	c.1152_1153ins	P (PP4, PM2, PVS1)
3	F	12 m	3	vestiges	LG:PE; RIT	6.72/0.21	0.36	17.6	c.671C>T:p.P224L	PrP (PS4, PM2, PP2, PP4)
4	F	4 y	F	yes					c.104G>A:p.G35D	
5	F	13 y	2	vestiges					c.1025C>T:p.S342L	PrP (PS4, PM2, PP2, PP4)
6	F	13 y	2		LM				c.1003A>C:p.T335P	PrP (PS4, PM2, PP2, PP4)
7	F	3 m	2	no	IT	3.93/1.22	1.3/13.3	41	c.848G>T:p.C283F	P (PS4, PM2, PM5, PP2, PP4)
8	F	16 y	2	no	IT	34/27.7	15.7		c.937C>T:p.R313C	
9	F	16 m	3	no	LM	0.87/0.2	0.1	20	c.1033C>A	
10	F	10 m	2	no	IT	3.6/1.5	0.22/24	40	c.70C>G:p.H24D	
11	F	17 m	3	no	RIT; LG:scrotum	10.5/2.16	0.5/4.51		c.37T>C:p.C13R	
12	F	15 y	F		RIT, LIT	85/43	9.5		c.72C>G:p.H24Q	P (PS4, PM2, PM5, PP2, PP4)
13	F	5 y	3	no	IT				c.251G>A p.R84H	
14	F	4 m	F	yes	LM	7.0/0.5	3.17/4.89		c.102+1G>T	
15	F	10 y	2	no	IT	5.54/0.3	/7.45	8.45	c.106T>C:p.F36L	PrP (PS4, PM2, PP2, PP4)
16	F	n/a	F						c.245-1G>T	
17	F	n/a	3						c.848G>A:p.C283Y	P (PS4, PM2, PM5, PP2, PP4)
18	F	10 y	2	no		10.3/3.2	10		c.732_733ins	P (PP4, PM2, PVS1)
19	F>M	6 w	4		IT	7.2/4.2	3.1/10	18	c.11391G>T	
20	M	2 m	4			5.03/1.69	4.0/7.8		c.244G>A:p.E81K	P (PS4, PM2, PM5, PP2, PP4)
21	M	4 w	3	no	scrotum	18.6/1.0	0.8	18.5	c.721C>T:p R241W	PrP (PS4, PM2, PP2, PP4)
22	M	4 y	4			1.64/0.1	0.09		c.C75A:p.Y25X	P (PP4, PM2, PVS1)
23	M	5 m	4		RIT; LG:scrotum	5.0/2.2	5.6/33	44	c.937C>T:p.R313C	
24	M	4 w	3		IT		4.3		c.937C>T:p.R313C	
25	M	13 y	3	no	RIT; LG:scrotum	14.4/5.32	15.9		c.T377A:p.M126K	P (PS4, PM2, PM5, PP2, PP4)
26	M	4 m	4		scrotum	7.2/4.2	2.63		c.C86T:p.T29M	
27	M	10 y	3	no	IT	1.7/0.2	0.26	31	c.591C>A:p.Y197X	P (PP4, PM2, PVS1)
28	M	14 m	3	no	IT	2.4/0.48	0.5/14		c.C909G:p.S303R	
29	M	15 y	3	no	LIT; RG:scrotum	9.1/8.1	26.2	42	c.G938A:p.R313H	
30	M	18 y	3	no	IT	8/5.5	18.3	0.6	c.251G>A:p.R84H	
31	M	12 m	3	no		1.1/0.2	0.2		c.1289G>A:p.S430N	
32	M	11 y	3			2.64/0.19	0.27		c.962G>T:p.G321V	
33	M	5 m	4		IT	2.3/0.2	0.03/4.6	60.4	c.251G>A:p.R84H	
34	M	18 y	4	no	RIT; LG:scrotum	9.6/6.9	11/20.8		c.951delC:p.H317QfsX17	
35	M	16 y	3	no	IT	8/5.5	15.4		c.951delC:p.H317QfsX17	
36	M	n/a	3						c.990G>A:p.E330E	P (PM2 PVS1 PP4)

Notes: y = years; m = months; w = weeks; T = testosterone; LG = left gonad; RG = right gonad; PE = pelvis; LIT = left-side inguinal testis; RIT = right-side inguinal testis; LM = labia majora; * = pathogenicity was determined for newly discovered variant mutations: P = pathogenic mutation; PrP = probably pathogenic (PP, PM and other values for pathogenicity calculation are specified in brackets [12, 13]); F>M = female sex changed to male; AMH > 28 ng/ml is normal for boys under 12 months [23].

Among the *NR1A1* mutations discovered for the first time, two (p.Y197X and p.Y25X) cause a stop-triplet development and two others (p.N385fs and p.L245fs) cause a reading frame shift; thus, their pathogenicity is beyond doubt.

Five of the variants not previously described are missense mutations (p.C283Y, p.C283F, p.H24Q, p.M126K, and p.E81K); just as one synonymic replacement that affects a splice site (E330E), these five were categorised as pathogenic; the remaining five mutations were categorised as probably pathogenic.

For the patients' phenotypical, hormonal and molecular genetic data, see Table 2.

DISCUSSION

Steroidogenic factor 1 is the key regulator of steroidogenesis and gonad differentiation. It was first identified in 1992 [3] as a transcriptional factor with tissue-specific expression that detects highly conserved regulatory motif in the proximal promoter region of genes coding steroidal P450-hydroxylases. Subsequent *in vitro* studies with adrenocortical cells found that NR5A1 also stimulates expression of the adrenocorticotropic hormone receptor (MCR2) and not just cytochrome-P450-dependent but all steroidogenesis enzymes [16].

An unexpected discovery was that NR5A1 is involved in formation and differentiation of adrenal glands and gonads. Thus, new-born *NR5A1-/-* mice with XY karyotype not only demonstrated male to female inversion and Müllerian duct derivatives but also had no gonads and no adrenal glands. Moreover, this model enabled the authors to establish that, in addition to gonads and adrenal glands agenesis, those mice displayed symptoms of primary disorder of gonadotropic function and agenesis of ventromedial hypothalamus nuclei [16]. By studying this phenomenon, Halvorson *et al.* showed that *NR5A1* also regulates the expression of β-subunit of LH and gonadotropin-releasing hormone receptor [17]. Thus, by now the role of NR5A1 in the formation, differentiation and function of the hypothalamus-hypophysis-adrenal and gonad systems has been established.

NR5A1 protein is comprised of 461 amino acids and is coded by *NR5A1* gene located on the long arm of chromosome 9 (9q33). The gene consists of 7 exons, of which 6 are coding. Several key domains are identified in the *NR5A1* structure: A-box – a DNA-binding domain with two zinc finger proteins which is responsible for the binding specificity; a less conserved hinge area; a ligand-binding area, and two domains responsible for the activation of NR5A1 function – AF1 and AF2. What regulates the transcription of *NR5A1* gene itself is still unclear. At present, there are two main candidate genes, *WT1* and *CBX2*, but so far no direct evidence to confirm such interaction has been obtained.

Given the data obtained through mice experiments, we originally searched for *NR5A1* mutations in patients who had 46,XY gonad dysgenesis combined with adrenal insufficiency. However, that phenotype turned out to be rare. One finds in the literature only a handful case reports describing 46,XY DSD with female phenotype, MDD and gonad dysgenesis combined with adrenal insufficiency where these were caused by *NR5A1* gene mutations [5, 8, 15]. This phenotype's infrequence might be explained, *inter alia,* by the fact that, similarly to the model tested in mice, mutations occurring in critical sections of the gene cause adrenal agenesis, which is not compatible with life.

In 2004, a number of independent research teams described heterozygous mutations in *NR5A1* as the cause of isolated 46,XY disorder of sex development without adrenal dysfunction [6, 18], and these suggest that haploinsufficiency occurs after *NR5A1* gene mutations.

It was subsequently found that heterozygous mutations in *NR5A1* are quite often (up to 20%) the cause of 46,XY DSD without PCAI [2, 7, 8].

To date, over 190 *NR5A1* mutations have been described, which are more or less evenly distributed over the gene's length [19]. Phenotype-genotype analysis found that the phenotype of patients with NR5A1 defects is variable and may manifest through various degrees of external genitalia ambiguity – from regular female to regular male morphology [7, 8, 19]. Given this diversity, Fabbri-Scallet *et al.* identified ten phenotypes associated with *NR5A1* mutations; they cover the entire range of conditions related to variations in these gene: pure gonad dysgenesis ± adrenal insufficiency; mixed gonad dysgenesis ± adrenal insufficiency; ovotesticular DSD; 46,XY-testicular DSD; isolated adrenal insufficiency; testicular regression syndrome; polycystic ovarian syndrome; male sterility [19].

In addition to variable phenotypic manifestations of one and the same mutation in non-related patients, various clinical implications do occur even in one and the same family. Philibert *et al.* described a patient with testicular hypoplasia and micropenis who had a heterozygous mutation (p.V355M) in *NR5A1* that was identified through analysing data of 24 anorchia patients. However, that person's twin brother who had a similar mutation did not show any abnormality at birth and his sex development during puberty period was normal [20]. This case suggests that there are other, yet unknown factors which affect the mutation allele expression and, accordingly, predetermine the phenotypic variability.

Given such polymorphous clinical manifestations, we found it interesting to analyse the frequency and phenotypic characteristics of, and the treatment tactics for, patients with *NR5A1* mutations within a cohort of Russian 46,XY DSD patients. As may be seen from the data we provided above, the frequency of pathological replacements in *NR5A1* within our group amounted to 11.6% of the total number of patients, which is commensurate with data published by international research teams.

As for the distribution of the child's sexes registered at birth, ambiguity of external genitalia was the main criterion. Thus, within the group with Prader 2 external genitalia ambiguity, all patients were registered as females, and in the Prader 4 group all but one were registered as males, whereas in the Prader 3 group the number of patients registered as females was approximately equal to that of males. This means that sex determination is an arbitrary choice.

Examinations of children during their minipuberty found a relative correlation between the levels of FSH and AMH, on the one hand, and the degree of external genitalia ambiguity, on the other. Thus, in children with elevated FSH levels the AMH level was lower, and their external genitalia were closer to female. High gonadotropin levels, typical for the "classical variants" of gonad dysgenesis in this period were not observed in any of the children we examined. During chorionic gonadotropin the response exceeded

20 nmol/L in two patients, which is rather typical for androgen insensitivity syndrome. Thus, no study of hormonal background in this age group may indicate NR5A1 disorders; however, such studies may point to the preservation of Leydig cells' steroidogenesis function, which may, in turn, help determine the child's sex to be registered.

Interesting data were obtained by examining patients who had reached puberty. In all five patients registered as males, regardless of the degree of external genitalia ambiguity at birth, spontaneous sex development and adequate development of secondary sex characteristics were observed. Even though this group was small, it is worth noting that at puberty stages 3–4 (ages 13, 15, 16), their testosterone levels were normal for the puberty period; however, at 18 they were below average values for Tanner 5 stage. At the same time, gonadotropin levels in these patients remained at or slightly over the upper limit of normal range. This may suggest a combination of primary and secondary hypogonadism and the explanation may be that correct NR5A1 function is important both on the gonad and the hypophysis levels. An important observation was that three patients registered as females with intact gonads developed masculinisation during puberty. Out of these three, two patients (no information was available on the third one) at the time of examination had testosterone levels at values normal for boys their age. This suggests preservation (restoration) of Leydig cells' steroidogenesis function, even though these patients had almost female external genitalia at birth (Prader 1–2), and it is important to keep this in mind as one selects the child's gender. A possible explanation may be that after birth, foetal Leydig cells are replaced with "adult" ones, which are another population [21]. Using mice models, it has been shown that the "adult" Leydig cells are less dependent on NR5A1 regulatory influence, since they have a higher expression of *NR5A2* gene, the liver homolog of NR5A1 receptor that partly substitutes for it [22]. Isolated cases of spontaneous masculinisation of phenotypic girls with *NR5A1* gene mutations have also been described by a number of international authors [9, 10]. Adachi *et al.* also provide their analysis of published data on histological examinations of tissue specimen obtained from gonads removed in such girls, since the risk of gonad malignancy in patients with 46,XY gonad dysgenesis is substantially higher than in those with other forms of DSD [10]. In eight cases out of nine, marked Leydig cell hyperplasia, combined with germline cell hypoplasia and aplasia, was observed. In none of those cases were any signs of malignancy described. However, Cool *et al.* described two patients who had their gonads removed during the puberty period; in one of them, *in situ* cancer was identified [9]. Most of our patients raised as girls had their gonads removed in their childhood, and in none of the cases were there any histological data pointing to malignancy. In three patients who had masculinisation during their puberty period, histological examinations showed no evidence of malignancy, either. Thus, data collected so far are yet insufficient to support an adequate assessment of risk of gonad malignancy in this group of patients.

CONCLUSION

Our findings provide evidence of a high frequency of *NR5A1* gene mutations in this group of patients and of the lack of any phenotypic or biochemical markers that could point to the aetiology of the disease prior to performing molecular genetic tests. More reliable findings can be achieved by continuing to monitor that group of patients, especially at the time they reach puberty.

ADDITIONAL INFORMATION

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Patient's consent. Informed consent was obtained from all examined patients; for those under 15, informed consent forms were signed by their legal representatives.

Authors' contribution. Every author has made a significant contribution to this research project and case report preparation and has read and approved the final version of the text prior to publication.

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