

# Novel Missense Mutation in Ligand-Binding Domain of AR Gene Identified in Patient with Androgen Insensitivity Syndrome from Ukraine

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## KEYWORDS

androgen insensitivity syndrome; androgen receptor; ligand binding domain; pathogenic mutation; bioinformatics

## ABSTRACT

Androgen insensitivity syndrome (AIS) is the most common disorder of sex development in people with karyotype 46,XY. Mutations in AR (androgen receptor) gene are found in most individuals with AIS. Exons 4-8, which encode LBD, were shown to be a mutation hotspot. **The aim** of this study was the search of mutations in the sequence of exons 6-8 which encode LBD of AR gene in patients with different clinical AIS phenotypes from Ukraine.

The investigated patients were 4 women with 46,XY karyotype, SRY-positive and clinical features of AIS (2 – CAIS, 2 – PAIS). Serum levels of T, LH, and FSH were quantified by electrochemiluminescence immunoassay (ECLIA) technology. Cytogenetic studies were performed on peripheral blood lymphocytes with further use of standard protocols of chromosomal analysis (GTG-banding). The presence of SRY sequence was confirmed by FISH with LSI SRY probe.

Direct Sanger sequencing of 6-8 exons was performed in patients and family members on the PCR products on the matrix of DNA samples isolated from peripheral blood lymphocytes.

Detected SNPs were analysed using gnomAD, VEP, MutationTaster, Human Splicing Finder, NetPhorest 2.1, Group-based Prediction System 5.0, and PhosphoPICK bioinformatical resources.

Modelling of mutant proteins based on available 3D models was conducted using the open source software UCSF Chimera 1.14rc.

We have detected 3 previously described mutations (missense mutation X:67722905 T>C (rs9332970) in PAIS patient, missense mutation X:67722943 C>T (rs886041132) in CAIS patient and, samesense mutation X:67723745 C>T (rs137852594) in PAIS patient). We determined these mutations as pathogenic using SIFT, PolyPhen, MutationTaster, Human Splicing Finder. Moreover the synonymous mutation X:67723745 C>T (rs137852594) detected in patient with PAIS was determined as mutation affecting processes of splicing.

In our study we have identified novel mutation X:67722884 T>G in CAIS patient and family members. This mutation was predicted as a pathogenic using aforementioned bioinformatical tools. STRUM calculations of the protein stability change caused by single-point mutation showed a destabilization effect of the Ile836Ser substitution  $\Delta\Delta G = -2.6$ . Possible aberrant phosphorylation analysis revealed the ability of MAPK family, Akt family, CDK1, CDK7, CDK9, PKC kinases to phosphorylate Ser836.

Results concerning the pathogenicity of X:67722905 T>C (rs9332970), X:67722943 C>T (rs886041132), X:67723745 C>T (rs137852594) mutations detected in patients with AIS from Ukraine obtained using bioinformatical resources SIFT, PolyPhen, MutationTaster, Human Splicing Finder correlate with previously published data concerning weaker binding of androgens in patients with the same mutations. This approves informativity of using such resources for mutation pathogenicity analysis.

Analysis of the ortholog proteins, subdomain structure, and aberrant phosphorylation of AR-LBD suggests novel X:67722884 T>G mutation to be pathogenic. Based on analysis of mutant protein modelling followed by assessment of free energy change using STRUM it was predicted that mutant protein binds androgens 460 times worse than wild type.

## INTRODUCTION

Androgen Insensitivity Syndrome (AIS) is a Disorder of Sex Development (DSD) with genetic etiology that

occurs at a frequency of 1 in 20,000 live births, and is the most common DSD in people with karyotype 46,XY. The phenotypes range from normal female genitalia in patients with Complete Androgen Insensitivity Syndrome (CAIS) to a wide range of ambiguous, undervirilized genitalia in patients with Partial Androgen Insensitivity Syndrome (PAIS). While PAIS is diagnosed neonatal, CAIS can only be detected in a healthy phenotypic woman in prepuberty and puberty [1]. According to the Danish Cytogenetic Central Registry, the prevalence of AIS was 4.1 per 100,000 women, with a median age of diagnosis approximately 7.5 years old. Mutations in the Androgen Receptor (AR) gene are found in most individuals with CAIS and a small number of individuals with PAIS. The AR gene encodes androgen receptor, which is a member of the nuclear receptor superfamily of ligand-dependent transcription factors, which also includes estrogen, progesterone, mineralocorticoid, and glucocorticoid receptors. AR is a single copy gene located at band Xq11-12 and is 183 kb long. It consists of 8 exons and relatively long introns. The first exon of the AR gene encodes the N-terminal domain (NTD); exons 2 and 3 each encode zinc finger, which together make up the DNA binding domain (DBD); the 5'-part of exon 4 encodes hinge domain; and the rest of the exons (4-8) encode the ligand binding domain (LBD) with activation function 2 (AF-2) [2]. The main function of the androgen receptor is the direct regulation of transcription. Binding of androgens to AR leads to a conformational change, allowing the transfer of the receptor from the cytoplasm to the nucleus, where it forms a homodimer. Then AR binds to a specific DNA sequence known as androgen response element (ARE). AR interacts with other proteins in the nucleus to modulate transcriptional regulation [3]. Androgen receptor undergoes phosphorylation, the patterns of which change after ligand binding. Phosphorylation of a certain amino acid residues has been found to affect various functions of the androgen receptor, such as activation of MAPK signalling cascade [4] [5] [6].

Currently more than 1000 mutations associated with AIS and prostate cancer have been described in the AR gene. Of those, about 600 were found in AIS patients, while 400 mutations fall in LBD.

**The aim** of this study was to research the spectrum and predicted of mutations in LBD of the androgen receptor gene among patients with androgen insensitivity syndrome from Ukraine.

## MATERIALS AND METHODS

### Patients

Ethical approval of this study was obtained from the committee on bioethics of the Institute of Molecular Biology and Genetics of National Academy of Sciences of Ukraine, protocol № 2 (30.04.2013). Informed consent was obtained from all patients and/or their parents. We have investigated the patients with clinical features of AIS and available relatives from four unrelated Ukrainian families. Serum levels of testosterone (T), luteinizing hormone (LH), and follicle stimulating hormone (FSH) were quantified by electrochemiluminescence immunoassay (ECLIA) technology on Cobas e 411 (Roche Diagnostics, Switzerland). Elecsys Testosterone II, Elecsys LH, Elecsys FSH kits were used according to the manufacturer's instructions.

### Genetic analysis

Cytogenetic studies were performed on peripheral blood lymphocytes using Microscope Nikon Eclipse Ci, Software: Lucia Karyotyping and FISH Software (Czech Republic) according to standard protocols of chromosomal analysis (GTG-banding, FISH - probes CEP, LSI (Probes: Yp11.3 – SRY; Yp11.1-q11.1 - DYZ3; Yq12 – DYZ1; CEP – DXZ1) Abbott Molecular, USA).

DNA from peripheral blood lymphocytes was extracted by a hydrolysis of cell lysates with proteinase K followed by phenol extraction.

The quality of DNA in samples was measured spectrophotometrically. PCR was performed using 5xHOT FIREPol® Blend Master Mix (Solis BioDyne, Estonia) according to the manufacturer's instructions, with primers described previously [7].

Visualization was performed on a 2% agarose gel, fragments of target length were excised from agarose, the PCR product was isolated and purified using Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Listed primers were further used for Sanger sequencing. Sequencing was performed using BigDye® Terminator Kits (Thermo Fisher Scientific, USA) on 3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA).

## Bioinformatics analysis and molecular modelling

Chromatograms were analysed and converted from .ab1 format to .fasta sequence using open source SnapGene 4.3.11 software (GSL Biotech LLC). The sequences were aligned using the Nucleotide Blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), against reference sequences of 6, 7, 8 exons of AR gene provided by Ensembl Genome Browser database (<https://www.ensembl.org/index.html>). Genome assembly - GRCh38, transcript - ENST00000374690.9. The frequency of found SNPs was determined using gnomAD v2.1.1 (<https://gnomad.broadinstitute.org/>). Impact of the mutation was assessed using the: Variant Effect Predictor (<https://www.ensembl.org/info/docs/tools/vep/index.html>), Varsome (<https://varsome.com/>), Human Splicing Finder (<http://www.umd.be/HSF/>) [8]. The probability of amino acid residues phosphorylation was verified using NetPhorest 2.1 (<http://www.netphorest.info/>), Group-based Prediction System 5.0 (<http://gps.biocuckoo.cn/>), and PhosphoPICK ([http:// bioinf. scmb.uq.edu.au/phosphopick/phosphopick](http://bioinf.scmb.uq.edu.au/phosphopick/phosphopick)).

To determine the potential pathogenic impact of polymorphisms on the protein structure, SIFT, PolyPhen and Mutation Taster values were used [9] [10] [11].

Three-dimensional coordinates of the protein structures and information concerning interactions with ligands were obtained from the RSCB PDB database (<http://www.rcsb.org/>). Modelling of mutant proteins based on available 3D structures was performed using UCSF Chimera 1.14rc open source software [12].

The protein stability change caused by single-point mutation was calculated with using web-server STRUM [13].

## RESULTS

In three cases of AIS family history was positive and in one case - negative. The karyotype of all investigated patients was 46,XY, SRY positive. The presence of SRY sequence was confirmed by FISH with Yp11.3 – SRY probe. Screening of the AR gene (exons 6–8) revealed mutations in three cases in exon 7 and one in exon 8.

**Patient UKR3201** 46,XY SRY+ was born with ambiguous genitalia: urogenital sinus, micropenis, both testicles are located in the split scrotum, the length of the blind ended vagina is 1.5 cm. Patient was registered in a female social sex. Laboratory tests: FSH 2.11 IU/L, LH 4.91 IU/L, total testosterone (TT) 2.15 ng/dl, free testosterone (FT) 2.71 ng/ml. The clinical phenotype of this patient was determined as PAIS. The family history of PAIS was confirmed. Pedigree anamnesis showed that proband's sibs also has PAIS. Patient UKR3201 was diagnosed with PAIS at the age of 1 year and 6 months.

Mutation X:67722905 T>C (rs9332970) was identified (Fig. 1).

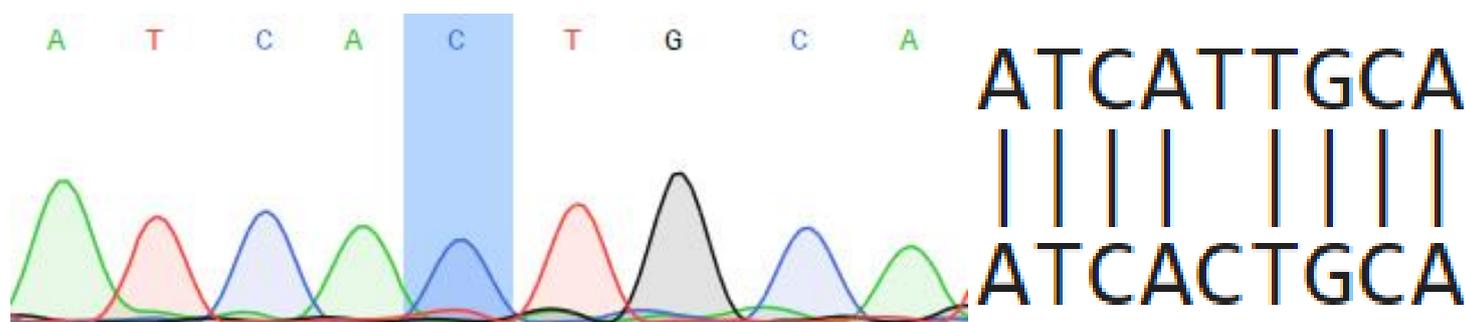


Figure 1. Partial electropherogram of exon 7 of androgen receptor (AR) gene.

Found substitution (missense mutation Ile843Thr) located in exon 7 (ligand binding domain). No allele frequency data is available. Pathogenicity was estimated using the following scores:

- SIFT – 0.001, damaging
- PolyPhen - 0.998, probably damaging
- Mutation Taster – 0.9999, disease causing.

**Patient UKR3301** 46,XY SRY+ was born with ambiguous genitalia: micropenis, glandular hypospadias, right testicle is located in the inguinal canal, left - in the hypoplastic scrotum. Patient was registered in a female social sex. The clinical phenotype of this patient was determined as PAIS and the diagnosis was made at birth. The family history was negative. Laboratory tests: FSH 159.9 IU/L, LH 2.7 IU/L, T 0,0002 ng/dL.

Substitution X:67723745 C>T (rs137852594) was identified. This transversion is a same-sense mutation (Ser889=) (Fig. 2).

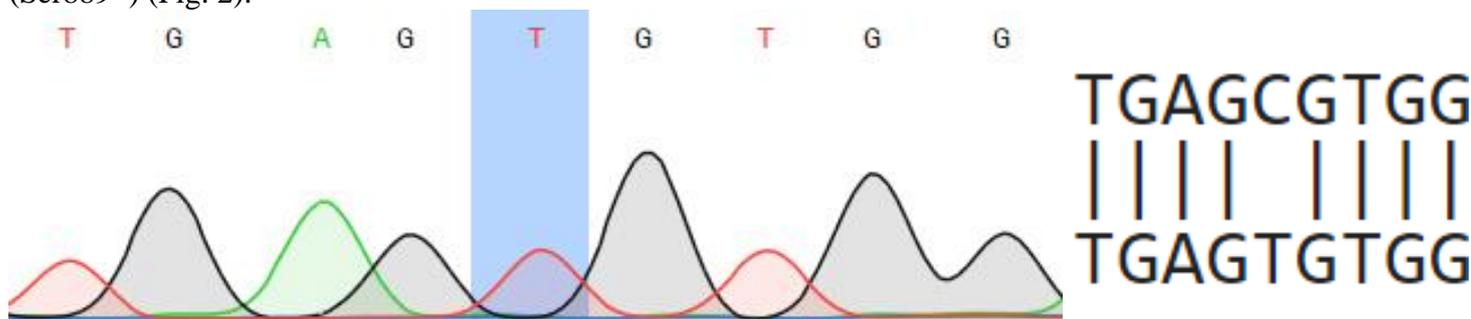


Figure 2. Partial electropherogram of exon 8 of androgen receptor (AR) gene.

Pathogenicity of silent mutation could be explained by the introduction of a new enhancer motif for the SRp55 spliceosome protein. Consequently, a new donor splice site appears at the end of the last exon 8, which causes the 3'UTR region translation, as predicted by Human Splicing Finder. Upon the activation of the cryptic splice site, the wild-type exon loses 67 nucleotides, and the protein becomes shorter by 25 amino acid residues (the last 8 amino acids are not native)

Thus, the resulting protein lacks fragment downstream of Met895. Particularly, the protein loses Ile899, which normally forms a binding pocket of the ligand-binding domain. This directly affects the ability to bind the ligand dihydrotestosterone (DHT) and causes a loss of function.

**Patient UKR0901**, 46,XY, SRY+, has started an examination at the age of 15 years for primary amenorrhea and delay of sexual maturation in female type. Patient has regular female external genitalia, 6 cm in length blind ended vagina, both testicles present in inguinal canal. The clinical phenotype of this patient was determined as CAIS. The family history of CAIS was confirmed. Pedigree anamnesis showed that mother's aunt also has AIS. Laboratory tests: FSH 4,8 IU/L, LH 4,91 IU/L, FT 0,0279 ng/dL. Mutation X:67722943 C>T (rs886041132) was found in patient UKR0901 (Fig. 3).

The detected substitution is a missense mutation (Arg856Cys) and located in exon 7 encoding ligand binding domain.

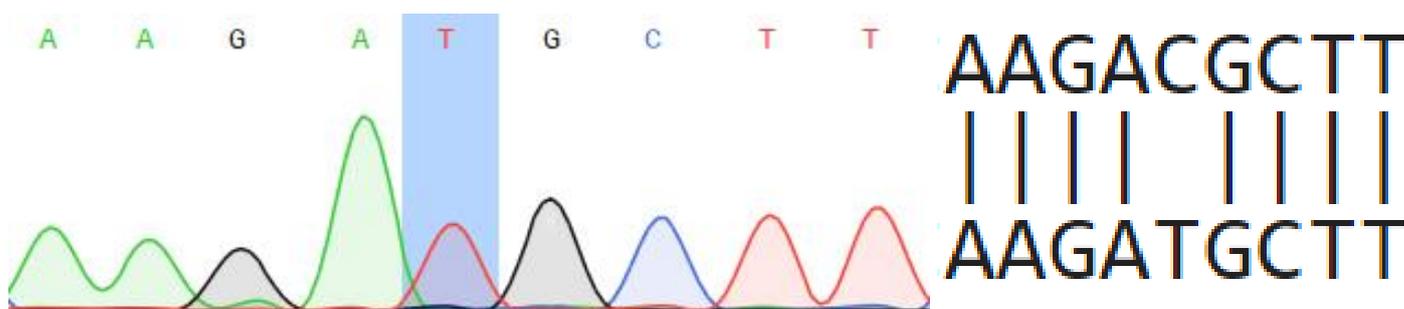


Figure 3. Partial electropherogram of exon 7 of androgen receptor (AR) gene.

This mutation was identified previously in patients from different countries [14] [15] [16]. No data is available on the allele frequency.

Pathogenicity was estimated using the following scores:

- SIFT – 0.001, damaging
- PolyPhen - 0.994, probably damaging
- Mutation Taster – 1, disease causing.

**Patient UKR1901**, 46,XY, SRY+, was born with regular female phenotype, female external genitalia, without signs of masculinization. Patient was registered in a female social sex. At the age of 2 years and 7 months patient was examined for bilateral “inguinal hernias”. The pedigree is burdened with AIS: grandmother (mother's mother) has two sisters: one has AIS, second is healthy but has AIS daughter. Ultrasonography revealed that both testicles are located in the inguinal canals. Size of the right is 28 x 9,5 x 16 mm and left one is 27 x 14 x 18 mm; the uterus,

cervix and both ovaries are not detected. The clinical phenotype of this patient was determined as CAIS. The family history of CAIS was confirmed. Laboratory tests: FSH 11.29 IU/L, LH 6.68 IU/L, TT 5.98 ng/dL (<7-20), FT 0,0081 ng/dL, dihydrotestosterone 483 pg/mL (24-368). Mutation X:67722884 T>G was identified in patient UKR1901 (Fig. 4). The databases Varsome, gnomAD, ARDB do not have data on this transversion, suggesting that this is a novel variant. This missense mutation is located in exon 7 (ligand binding domain) and results into the substitution Ile836Ser.

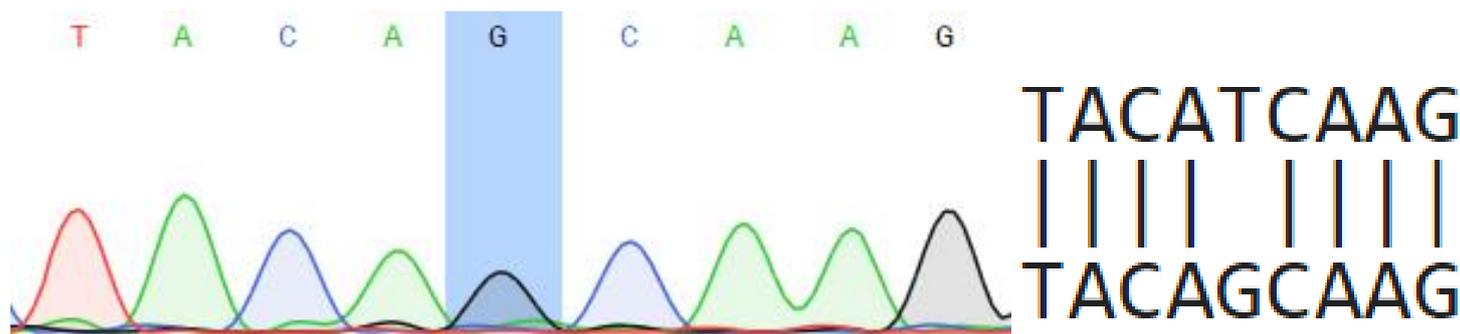


Fig. 4. Partial electrophoregram of exon 7 of androgen receptor (AR) gene.

Sequencing was also performed on family members of the proband, shown in Figure 5. It is worth noting that cousin of proband's mother (person III:5), who had also been diagnosed with CAIS, is hemizygous carrier of the same mutation. Maternal grandmother (person II:2), mother (person III:2) and healthy sibling (person IV:2, 46,XX girl) are heterozygous carriers of identified X:67722884 T>G mutation.

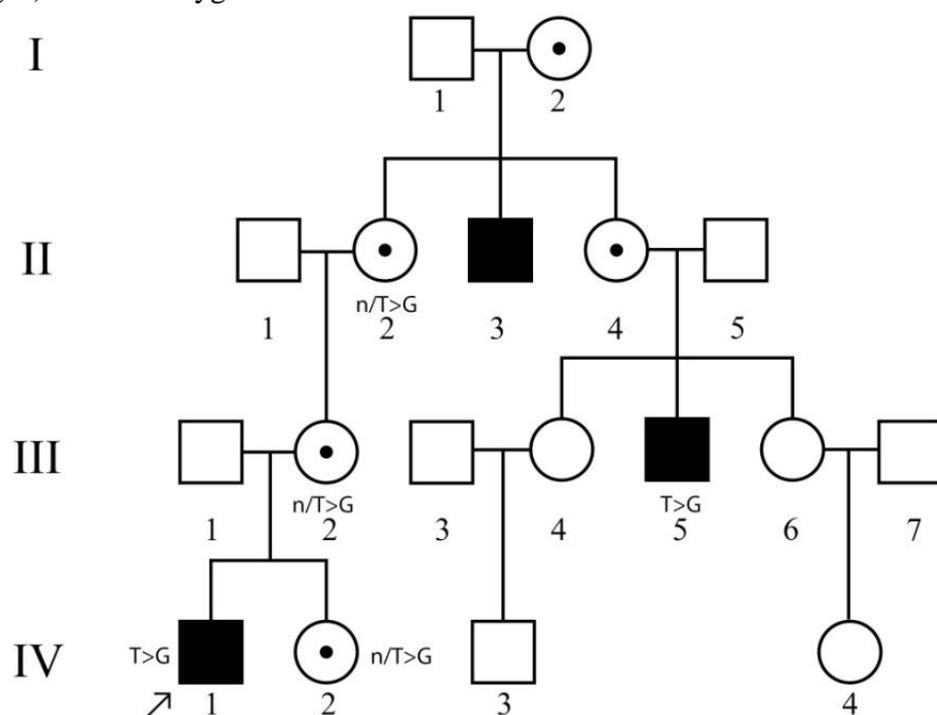


Fig.5 Pedigree of patient UKR1901 with identified novel X:67722884 T>G mutation in exon 7 of AR gene

Pathogenicity was confirmed using three different pathogenicity scores:

- SIFT - 0, damaging
- PolyPhen - 0.988, probably damaging
- Mutation Taster - 1, disease causing.

Evaluation of phosphorylation pattern changes suggests that the following known AR-specific kinases act upon mutant Ser836: PKC kinase (NetPhorest); MAPK family kinases (Group-based Prediction System); CDK1, CDK7, CDK9, kinases from the Akt and MAPK families (PhosphoPICK).

This amino acid substitution occurs in helix 9 of ligand-binding domain, which is one of the target regions in the treatment of prostate cancer with androgen receptor antagonists. This fact, and the close location of the side radical of Ile836 to Phe916 required for the binding of androgens is indicative of the pathogenicity of this mutation [17].

Considering the substitution of amino acid radicals, it can be noted that native Isoleucine has a much longer nonpolar side chain, compared to the shorter polar Serine, which also has a hydroxyl group –OH, which causes changes in protein's hydrophobicity profile (Fig. 6). Predictive modelling of Ile836Ser substitution using PDB ID: 2PIX structure as a template was conducted, to measure changes in side chains distances. Analysis of the substitution on the 3D model structure of showed that the distance between the side radicals Ile836 and Phe916 is 3.94 Å, while the distance Ser836-Phe916 is 1.58 Å more and is 5.52 Å (Fig. 7).

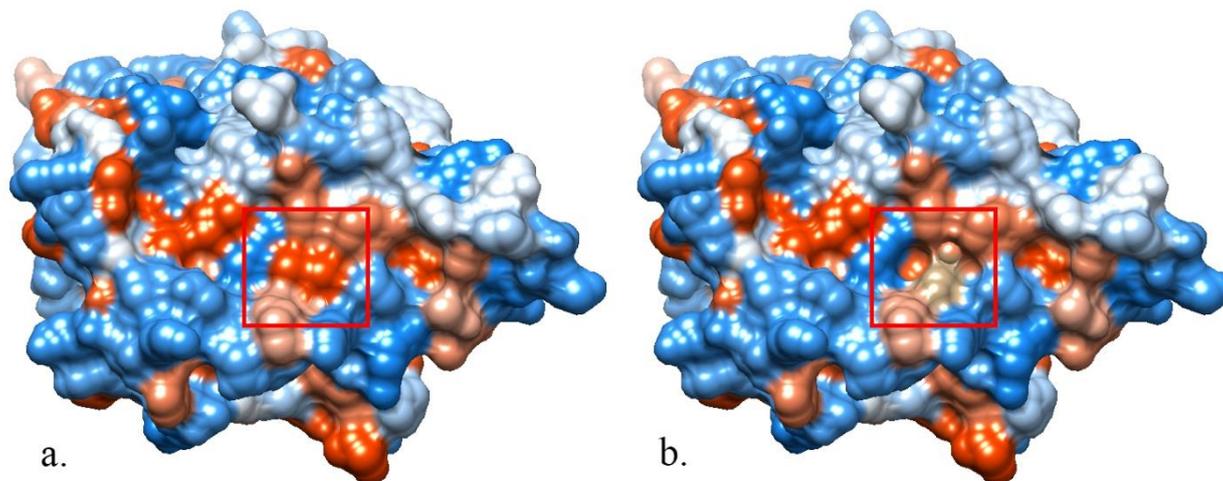


Fig. 6. Hydrophobicity profile changes between wild type AR (a) and Ile836Ser mutant (b). (Red - hydrophobic region, blue - hydrophilic) PDB ID: 2PIX

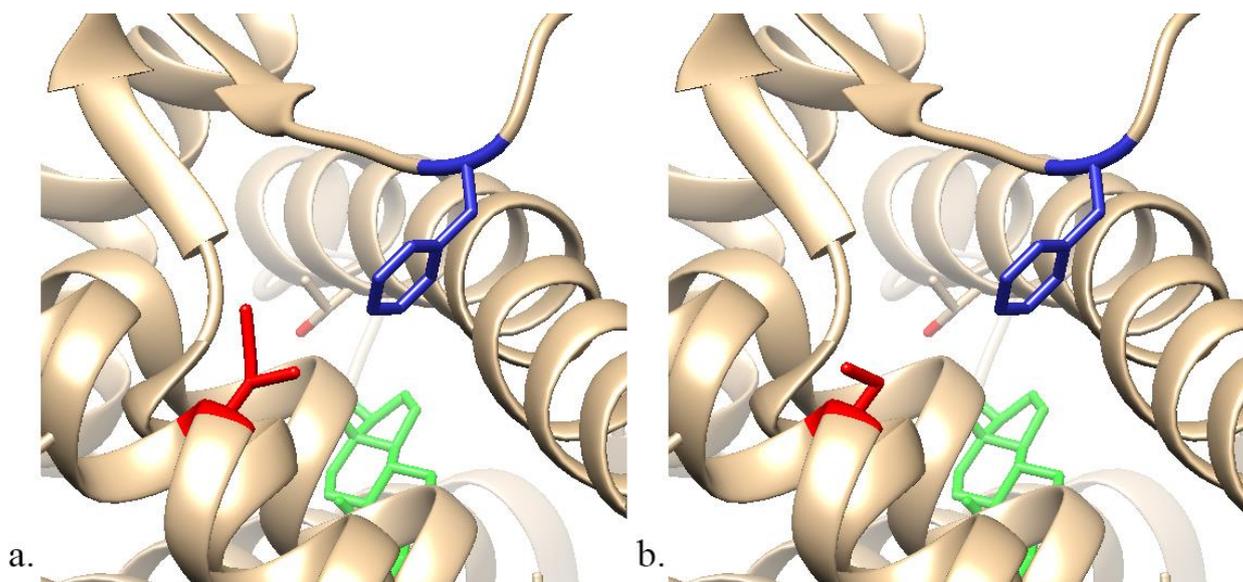


Fig. 7. Side radicals changes between wild type AR (a) and Ile836Ser mutant (b). (Red - amino acid 836, blue - Phe916, green - DHT) PDB ID: 2PIX

STRUM calculations of protein stability change caused by single-point mutation showed destabilizing impact of Ile836Ser substitution. While  $\Delta\Delta G < 0$  result is supposed to be destabilizing, and  $\Delta\Delta G < -1$  considerably destabilizing, our substitution showed result  $\Delta\Delta G = -2.6$ .

Molecular dynamics modelling is the next step in determining the nature of the pathogenicity of Ile836Ser substitution.

## DISCUSSION

We performed a bioinformatical analysis of mutations detected in AIS patients from Ukraine. Three missense mutations in exon 7 and one synonymous mutation in exon 8 (both coding LBD) were classified as pathogenic by SIFT, PolyPhen, MutationTaster, Human Splicing Finder, whose algorithms take into the account amino acid conservation, location of an amino acid in functionally important regions of the protein, and the existence of studied

SNP in multiple databases. Quantitative and qualitative data on ligand binding were obtained earlier for patients with X:67722905 T>C (rs9332970), X:67723745 C>T (rs137852594), X:67722943 C>T (rs886041132) mutations. In a PAIS patient (UKR3201) with X:67722905 T>C (rs9332970) Ile843Thr mutation all used resources (SIFT, PolyPhen, MutationTaster) evaluate this substitution as pathogenic, what correlates with experimental results of  $B_{\max}$  (total receptor concentration in the tissue sample)  $-7 \times 10^{-12}$  mol/g protein, and  $K_d$  (equilibrium dissociation constant)  $-0.35 \times 10^{-9}$  M (normal  $K_d = 0.08 \times 10^{-9}$  M). This argues for a reduced ability of the protein to bind androgens. Noteworthy, this mutation was found previously in a patient with CAIS phenotype [18].

Such a difference in the severity of AIS phenotype in patients bearing the same mutation can be explained by the possible length variation in the polyG and polyQ tracts of AR. The length of the  $(GGN)_n$  repeat in exon 1 has been shown to modulate AR transactivation and translation [19] [20].

In the case of CAIS patient (UKR0901) with X:67722943 C>T (rs886041132) Arg856Cys mutation all used in our study bioinformatical resources (SIFT, PolyPhen, MutationTaster) evaluate this substitution as pathogenic, what also correlates with previously published experimental results concerning such a mutation of  $B_{\max} < 10 \times 10^{-18}$  mol/g DNA (normal parameters in the culture of genital skin fibroblasts  $630 \times 10^{-18}$  mol/g DNA) and  $K_d = 0.125 \times 10^{-9}$  M (normal value  $K_d = 0.08 \times 10^{-9}$  M), which implies a reduced androgen-binding ability [21].

In our study, bioinformatical estimation of X:67723745 C>T (rs137852594) Ser889= mutation with Human Splicing Finder found in PAIS patient (UKR3301) showed introduction of a new enhancer motif for the SRp55 spliceosome protein, which results in modified mature mRNA transcript. In previous studies, in PAIS patient with this mutation no binding of androgens was found ( $B_{\max} = 0$ ,  $K_d = 0$ ) [22]. In addition, appearance of another, significantly shorter, aberrant transcript (5.5 kb vs. normal 10.5 kb) was shown, as a result of effect of this substitution on splicing [23]. It is not a unique example of splicing perturbation by a silent mutation impact. In 2017, another synonymous mutation was found in exon 1 (N-terminal domain) which resulted into CAIS in two unrelated patients from Brazil [24].

Analysis of novel mutation X:67722884 T>G Ile836Ser detected in CAIS patient UKR1901 suggests several possible mechanisms of its pathogenicity. Firstly, we determined this mutation as pathogenic using SIFT, PolyPhen, MutationTaster. It is important to note that the replacement of Isoleucine's longer nonpolar side chain by Serine's shorter polar side chain destabilizes the protein, as was shown by STRUM – instrument, assessing protein stability changes.

3D modelling of Ile836Ser mutation based on a protein crystal structure showed changes in hydrophobicity profile (Fig. 6) and enlarged distance between amino acid 836 and Phe916 (Fig. 7), which is important for androgen binding. Phe916Ala mutant was shown to bind androgens 460 times worse than wild type protein.

Moreover, AR is known to undergo post-translational phosphorylation (before and after ligand binding), which affects the protein functions depending on the site of phosphorylation. Kinases from MAPK and Akt family; kinases CDK1, CDK7, CDK9, PKC are capable of phosphorylating Ser836.

Androgens are known to rapidly activate kinase signalling cascade and modulate intracellular level of calcium ions. Binding of dihydrotestosterone to AR allows the receptor to interact and activate the Src tyrosine kinase. The activation of Src leads to the phosphorylation of EGFR (Epidermal growth factor receptor). It has also been shown that AR rapidly is (within 5 minutes) phosphorylated after the interaction with dihydrotestosterone, which leads to the activation of ERK and CREB kinases within 1 minute. This is necessary to maintain spermatogenesis in Sertoli cells. It was shown that it takes dihydrotestosterone at least 45 minutes to induce transcriptional activity in addition to time required for protein synthesis. Thus, the appearance of a new phosphorylation site which was predicted in our study by NetPhorest 2.1, Group-based Prediction System 5.0, and PhosphoPICK resources may affect aforementioned functions of AR.

## CONCLUSIONS

Results concerning the pathogenicity of X:67722905 T>C (rs9332970), X:67722943 C>T (rs886041132), X:67723745 C>T (rs137852594) mutations detected in patients with AIS from Ukraine obtained using bioinformatical resources SIFT, PolyPhen, MutationTaster, Human Splicing Finder correlate with previously published data concerning weaker binding of androgens in patients with the same mutations. This approves informativity of using such resources for mutation pathogenicity analysis.

A novel substitution, X:67722884 T>G, was identified in a Ukrainian patient with a family history of CAIS.

Analysis of the ortholog proteins, subdomain structure, and aberrant phosphorylation of AR-LBD suggests mutation X:67722884 T>G to be pathogenic. Based on analysis of mutant protein modelling followed by

assessment of free energy change using STRUM it was predicted that mutant protein binds androgens 460 times worse than wild type.

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