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Review of a Dissertation for the degree of Doctor of Philosophy of Zofia Kolesińska titled „IDENTIFICATION OF THE GENETIC BACKGROUND IN 46,XY DISORDERS OF SEX DEVELOPMENT” prepared at the request of the Dean of Faculty of Medicine I of Poznan University of Medical Sciences

Introduction

According to the consensus reached in Chicago in 2005 differences and disorders of sex development (DSD) became subdivided into three main categories: sex-chromosome DSD, 46,XX DSD, and 46,XY DSD on the basis of the result of karyotyping.

As the aetiology seems to be obvious in the sex-chromosome DSD subgroup as well as in cases with 46,XX karyotype who are diagnosed usually with congenital adrenal hyperplasia, more than a half of patients with 46,XY DSD do not receive the definitive genetic diagnosis. If the pathogenesis is being considered, one can divide 46,XY DSD into three main subgroups: disorders of gonadal development (DGD), disorders of androgen synthesis (DAS) and disorders of androgen action (DAA).

As many genes are involved in formation of the bipotential gonad and its subsequent differentiation into the testis, the elucidation of the genetic background in DGD is much more challenging in comparison to other 46,XY DSD subgroups. Indeed, one can identify the causal mutation in less than half of reported cases. In the context of above data the undertaken topic of presented Dissertation is fully justified.

Information on Dissertation for the degree of Doctor of Philosophy

Dissertation for the degree of PhD counts 78 pages and has typical layout of scientific work supplemented with *List of Abbreviations, List Tables and Figures*.

The *Introduction* is a wide-ranging chapter with eight parts in which the Author presents clearly, in logical order and in detail data from the literature sequentially referring to *Disorders of Sex Development, Male Sex Determination and Prenatal Differentiation, Hypothalamic-pituitary-gonadal axis, Disorders of Gonadal Development, Disorders of Androgen Synthesis, Disorders of Androgen Action, New gonadal markers: AMH and inhibin B and Next Generation Sequencing in DSD*.

In the *Aim of the study* PhD Candidate specified four main purposes of the project

1. to perform thorough clinical, biochemical and radiological assessment in patients with 46,XY DSD.

2. to identify the genetic background of 46,XY DSD with the use of massive parallel sequencing technology.
3. to establish a link between the results of clinical, biochemical and radiological investigations and the targeted gene sequencing.
4. to develop an algorithm of integrated diagnostic approach taking into consideration the targeted NGS 46,XY DSD gene panel.

Subsequently the *Study group* and *Methods* are described scrupulously.

The study was approved by the local medical ethical committee (No 505/13) and all participants, parents as well as children over 16 years, signed the informed consent forms. The study group consisted of 35 patients diagnosed with 46,XY DSD according to the consensus reached in Chicago and referred to the Department of Paediatric Endocrinology and Rheumatology for further investigations. The younger presented with atypical genitalia, while adolescents complained of primary amenorrhoea. Diagnosis was established based on appearance of external genitalia using external masculinisation score (EMS) in those patients diagnosed at a younger age. Among 35 participants, 21 (60%) patients were raised as boys, the remaining 14 (40%) were raised as girls. There were 8 patients diagnosed with syndromic 46,XY DSD, defined as the presence of atypical external genitalia and multiple associated conditions.

The PhD Candidate based on UK guidelines established the preliminary diagnosis using clinical, biochemical and radiological data. The only difference was the nosological entity of non-specific disorder of undermasculinisation (NSDUM), which was postulated to be used for patients with a preliminary diagnosis of partial androgen insensitivity syndrome (normal hormonal activity of testes in a boy with atypical genitalia) without proven mutation in the AR gene. Standard hormonal measurements were performed using commercial kits. The assessment of internal genitalia (presence or absence of uterus, localization of undescended gonads) was done as a part of a routine diagnostic procedure during abdominal ultrasound performance and/or during gynaecologist assessment performing transabdominal examination.

Dr Zofia Kolesińska precisely described genetic investigation procedures. Following a thorough literature and database search she has selected a list of 54 genes for evaluation. These include genes in which mutations involved in the aetiology of 46,XY DSD had previously been described. Fifty of the selected genes were implicated in 46,XY DSD by multiple reports of independent families with similar phenotype. If the gene was reported in a single human case or family, she selected for the present study only those genes for which reports demonstrated sufficient experimental data, in accordance with the 'Guidelines for investigating causality of sequence variants in human disease'. The less confident genes implicated in the pathogenesis of DSD were as follows: *CBX2*, *FGFR2*, *WWOX*, and *TSPY1*. In some of the selected genes the type of causative genetic change was not predicted to be identified by the NGS technology, particularly deletions encompassing *DMRT1* and *WWOX* genes and duplications of *NR0B1* and *WNT4*. When the AR gene was considered, the number of CAG as well as CGG repeats were also analysed. NGS procedures and bioinformatic analysis were performed at the Laboratoire de Diagnostic des Maladies Génétiques in Lausanne in Switzerland. The PhD Candidate follows the rules defined by the American College of Medical Genetics and Genomics (ACMG) to weight variant evidence. The identified mutations she classified into three categories: pathogenic variants (PV), likely pathogenic variants (LPV), and variants of unknown significance (VUS).

The *Results* section dr Kolesińska divided into five parts: 1. Preliminary diagnosis based on clinical, biochemical, and radiological findings; 2. General aspects of identified mutations with targeted 46,XY DSD gene panel; 3. Identified variants in the light of

integrated phenotype and genotype picture; 4. The concordance between the preliminary diagnosis and identified variants, 5. The concordance between the preliminary diagnosis and identified variants. Most of the results she presented in well designed tables and figures with appropriate descriptions

By analysing clinical, biochemical, and radiological findings dr Kolesińska identified 9 patients diagnosed with DGD (5 boys assumed with TRS and 4 patients raised as girls with CGD); 4 patients with DAS (one raised as girl); 14 patients with a diagnosis of DAA (six boys suspected of NSDUM, seven girls of CAIS and one of PAIS) and 8 children that fulfilled criteria of syndromic DSD. Their clinical and biochemical phenotype was very detailed and preciously described in table 2 and 3.

The genetic analysis led to the identification of 22 mutations in patients raised as boys and 19 mutations in patients raised as girls. In the subgroup of patients raised as boys, there were 15 missense mutations, two deletions of 1 and 7 nucleotides respectively leading to a frameshift change, one in frame duplication of 6 nucleotides, one mutation impacting splicing, and three significant expansions of CAG repeats (only CAG repeats >27 were reported). Among patients raised as girls, there were 10 missense mutations, one deletion of 7 nucleotides and one duplication of 1 nucleotide, both leading to a frameshift change, two nonsense mutations, four mutations impacting splicing (one in 3'utr and three in intronic regions), and only one significant expansion of CAG repeats.

In boys, the mutations were most frequently identified within the *AR* gene (n=4), followed by the *ATRX* gene (n=2) and the *POR* gene (n=2). In girls, the gene that harboured the highest number of mutations was similarly the *AR* gene (n=8). Furthermore, both *CYP17A1* and *HSD17B3* genes harboured two mutations. When analysing the group as a whole, the gene harbouring the highest number was the *AR* gene with 12 changes, followed by the *HSD17B3* and the *POR* genes with three changes in each, and finally the *ATRX*, *CYP17A1*, and *TACR3* genes with two changes in each.

Dr Kolesińska compared the identified mutations to the clinical, biochemical, and radiological findings, inheritance pattern, as well as to the literature and databases' reports, in order to weigh evidence of each variant and attribute its particular status of PV, LPV, or VUS. She found a clear difference between the diagnostic rate between patients raised as boys compared to patients raised as girls. She assumed that the definitive diagnosis was reached when PV or LPV was identified. Accordingly, the diagnostic rate was 14.3% in the subgroup raised as boys (3 LPV), while in the subgroup raised as girls it reached 64.3% (8 PV and 2 LPV in 14 patients; 2 PV were identified in 1 compound heterozygote). In the subgroup raised as boys there were 12 VUS identified, and as dr Kolesińska highlighted, further studies may lead to the increase of the diagnostic rate among boys.

When the aetiology of DSD was considered in the subgroup raised as boys, the same diagnostic rate was reached among boys with suspected DAA and DAS. Furthermore, there were 2 VUS identified in boys with DAA, 3 VUS in boys with DAS, and as many as 7 in boys with syndromic DSD. Therefore, as the Author emphasized, higher diagnostic rate may be seen when additional studies are performed as a number of the VUS variants may be subsequently reclassified. Interestingly, there were no variants identified within the subgroup of boys with DGD. The analysis of girls revealed similar results; the highest diagnostic rate was reached within the subgroup diagnosed with DAA, as all patients reached a definitive genetic diagnosis (6 PV and 2 LPV) and in the subgroup diagnosed

with DAS (2 PV in one patient that was found compound heterozygote). The lowest diagnostic yield was found in girls with DGD (3 VUS).

The PhD Candidate's data for the whole group of 46,XY DSD patients regardless the sex assignment clearly showed that the genetic diagnosis was reached with highest probability in the subgroup of children suspected for the improper function of the receptors of androgens, while for children with a preliminary diagnosis of DGD (i.e. namely testicular regression syndrome and complete gonadal dysgenesis), and those with associated conditions, no definitive genetic diagnosis was possible.

Dr Kolesińska's analysis confirmed that the concordance between the preliminary diagnosis and the identified variants was significant ($p=?$) in DAA and DAS subgroup, less significant in syndromic cases, and absent in DGD cases. Her study revealed that when the specificity of the genes was considered, the less specific DSD genes were the known CHH genes. Variants in the known CHH genes were identified in patient with CAIS, patients with CGD, and in those diagnosed with syndromic DSD. Moreover, in her cohort of 46,XY DSD patients, there were 5 (14.3%) cases harbouring mutations in more than one of the DSD genes. Interestingly, almost all patients had a combination of variants in the AR gene and at least one other gene. This shows that targeted NGS gene panel also evaluates additional factors that may modify particular phenotypes as the oligogenic nature of DSD.

In the context of presented results, one can acknowledge, that the performance of targeted gene panel analysis in patients with this heterogeneous condition reached a satisfactory level of detectability, while in cases that were identified with VUS, it constitutes an excellent starting point for further analysis.

The successive points of the *Results* refer precisely to set goals.

In the *Discussion* the Candidate confronted her results with data from appropriately selected literature. A maturity of PhD Candidate as a researcher were manifested through critical verification of received results and by showing the limitations of the study.

Dr Kolesińska emphasized this was the first study that characterize genetic profile of 46,XY DSD Polish cohort. Despite small size of the study group, it led to the identification of recurrent mutations, thus it strengthened the contribution of ethnicity into the genetic background of DSD that should be always taken into consideration.

Based on the results the PhD Candidate accurately expressed the *Conclusions* which answered for the previously formulated aims. Next chapters are *Summary* in English and Polish version, *References* and *List of Tables and Figures*.

Because of reviewer's obligations I would like to add some comments/questions to the presented dissertation:

Patients and Method: It is desirable to present the information about the time of patients' recruitment to the study.

Results:

- Even if the group of patients is small in numbers for some comparisons the statistical analysis is obligatory and should be presented even with non-significant differences.

- Reading the *Results* one may have the impression that it is more like discussion – there are comments and discussion in the Results chapter, eg. "... that was represented by only one case ID 34, there was a mismatch between the preliminary diagnosis and the identified PV. The selected variant was found in the NR5A1 gene, which is traditionally perceived as a candidate gene in gonadal dysgenesis with or without adrenal failure [58]. However, it has been proved that the phenotype is much broader including proper hormonal function in the affected patients [59]. Interestingly, recently the same mutation was found in three unrelated patients with 46,XX ovotesticular DSD, thus it has been hypothesized that p.Arg92Trp results in decreased inhibition of the male developmental pathway through downregulation of contrary genes shifting the balance in favour of testicular development [60, 61]. Therefore, it is believed that this recurrent mutation can act as a molecular switch in human sex development leading to undermasculinisation of 46,XY patients and overmasculinisation of 46,XX patients [60, 62]."

Tables & Figures:

- Table 8 – it will be easier to follow by giving the ID of the patients instead of number of patients.
- Figure 10- lack of legend
- Figure 2 – lack of explanation of "c"

Minor errors:

- repeatedly incorrect record of numbers eg. "14,3", "28,6" –it should be 14.3, 28.6 etc,
- minor errors: "polish", "english"
- lack of explanation of "CNV" abbreviation

Points for discussion:

- The most difficult aspect in the management of DSD is certainly the sex assignment. There are attempts to identify the factors which influence this decision, including diagnostic-specific psychosexual development, genital appearance, fertility potential, risk of gonadal tumour development, surgical options, the need for hormone replacement therapy, with the regard to family opinion and their culture and religious framework. What is the place of genetic analysis nowadays?
- Do you analyze routinely the steroid profile in the urine of DSD patients? Is steroid profile in the urine (24H collection) useful in diagnostic procedures of 46,XY DSD?
- There are different protocols for hCG test. Are there any advantages of that used in your study?

Conclusions:

In summary, I read the PhD thesis provided by Dr Kolesińska with great interest. The work provided is clearly ambitious and describes very interesting results, with high level of novelty. The study by Dr Kolesińska was very well designed and perfectly conducted. The PhD Candidate set goals which she realized by thorough analysis and finally defined

the relevant conclusions. It needs to be highlighted that presented study is unique in Poland and dr Kolesińska presents the readiness to continue it.

The reviewed Dissertation of dr Zofia Kolesińska titled „IDENTIFICATION OF THE GENETIC BACKGROUND IN 46,XY DISORDERS OF SEX DEVELOPMENT” fulfills all requirements posed on theses aimed for obtaining a PhD degree in agreement with conditions defined in article 13, paragraph 1, Act of 14 March 2003, Law and Academic Degrees and Title and Degrees and Title in the Arts (Dz.U. Nr 65, poz. 595, późn. zm.). Moreover, I ask the High Faculty Council of Medicine, Poznan University of Medical Sciences to award Dissertation of dr Zofia Kolesińska with appropriate prize or distinction.

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