Integrated analysis of whole-exome sequencing for molecular genetic diagnosis of 46,XY disorders of sex development

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Integrated analysis of whole-exome sequencing for molecular genetic diagnosis of 46,XY disorders of sex development

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<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Integrated analysis of whole-exome sequencing for molecular genetic diagnosis of 46,XY disorders of sex development

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Purpose: To determine the etiologic diagnosis through whole-exome sequencing (WES) within Korean patients with disorders of sex development (DSD) cohort.

Methods: Among patients with ambiguous genitalia who visited the department of pediatric endocrinology in a tertiary university hospital, 80 patients with 46,XY DSD who underwent WES were included. The median age was 2 years (range, 1 month-18 years).

Results: WES analysis identified total 20 variants of 11 genes from 19 patients. The diagnostic yield of pathogenic or likely pathogenic genetic variants were 15% (12/80). We identified 12 novel variants from 8 genes known to induce DSD: *NR5A1* (p.R84C, p.R84L, p.R87L and p.R313H), *SOX9* (p.R117Q), *AR* (p.R569H), *FGFR1* (p.V184M and p.P633L), *MAMLD1* (p.474-477 del), *ZFPM2* (p.H320fs), *NR0B1* (p.L294P), and *NR0B2* (p.G99fs). Five novel variants were also identified from three known genes with different phenotypes: *BMP15* (p.L263delinsHL), *POF1B* (p.R339W and p.S295X), and FRAS1 (p.R2978L and p.Y2273X).

Conclusion: This is the first report of applying WES in a large Korean cohort of patients with 46,XY DSD. WES was useful in identifying causative variants of 46,XY DSD patients.

Key words : 46,XY disorders of sex development, whole exome sequencing, genetic testing

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I. INTRODUCTION

The challenge that physicians face with ambiguous genitalia is the extensive diversity of underlying etiology. Disorders of sex development (DSD) is defined by congenital conditions in which chromosomal, gonadal, or anatomic sex is atypical[1]. In addition to the demand for multi-level approach, complexity of developmental process itself further complicates the diagnosis of DSD. Disturbance of any one or combination of each step of sex development may have potential causality for clinical manifestations. This explains why the incidence of DSD varies so widely from one in 200-300 to 4,500-5,500 births in previous reports[2,3]. DSD is a genetically heterogeneous disease entity with low clinical diagnostic rate. Clinically, the standardization of phenotypes is absent and analytically, only a limited number of genes causing DSD have been identified to date.

Because identical phenotypes may arise from different genetic etiologies, genetic testing has been a mainstay in diagnosis of DSD. The advancement of genetic testing has continued in an increasingly molecular and submicroscopic fashion. Karyotyping visualizes alterations in chromosomes[4]. Fluorescence in situ hybridization (FISH) is a cytogenetic technique which detects loss or gain of a specific segment of chromosomes[5]. Comparative genomic hybridization (CGH) analyzes variation in number of certain gene copies[6]. Real-time polymerase chain

reaction (RT-PCR) tests for changes in expression of specific genes[7]. Sequencing of DNA identifies pathogenic variants of a gene[8]. Among several clinically available gene testing methods, next generation sequencing (NGS) is a newer, widely used method to identify the genetic etiology by virtue of its advantages of rapid and high-throughput detection of causative genes. Currently, targeted panel NGS has become the preferred method in clinical diagnosis due to its lower costs, shorter detection cycle, greater sequencing depth, and lower data processing. Compared with targeted panel NGS, WES has advantages of more improved accuracy and detection of novel genes causing disease[9]. Exomes are protein-coding regions of all genes approximately 20,000 in number. Although the exome consists of only about 1% of the whole genome, it bears 85% of mutations responsible for diseases. Focusing on the regions where majority of the disease-causing genes are concentrated enhances the chance of improving diagnostic yield of a genetically heterogeneous disease entity such as DSD. While targeted NGS panel tests are limited to detecting only the known variants for DSD which are already included in the panel, WES may enhance diagnosis by detecting previously unknown, new variants related to DSD. Because as few as less than 15% of patients with DSD are given accurate genetic diagnosis, detecting newer variants with WES is of significant importance in improving diagnosis of DSD[10]. This study was conducted to assess the diagnostic yield of WES in 46,XY DSD patients with ambiguous genitalia and explore its potential for finding novel variants.

II. MATERIALS AND METHODS

1. Patient selection and study design

Among patients with atypical genitalia who visited the department of pediatric endocrinology in a tertiary university hospital from May, 2015 to March, 2017, 80 patients with 46,XY DSD who underwent whole-exome sequencing (WES) were included. The preliminary diagnosis of DSD was

based on clinical manifestations, laboratory and imaging studies. The inclusion criteria were as follows: 1) ambiguities of external genitalia; and 2) other clinical manifestations of DSD 3) 46,XY Chromosome karyotype. Exclusion criteria was DSD patients who did not have WES. Trio test was performed at physician's discretion. The medical records and laboratory results were retrospectively analyzed. This study was reviewed by the Institutional Review Board (IRB No. 4-2015-0052). All identifiable personal information of the included patients was anonymized during analysis. Informed consents for all patients were obtained from their parents, and patients as appropriate.

2. Endocrine investigations

In addition to thorough physical and perineal exam, blood tests including complete blood count with differential, chemistry profile, and hormonal levels were obtained at each visit. In case of impalpable gonads as well as imperceptibility of gonads on imaging were considered as gonadal dysgenesis. Human chorionic gonadotropin (hCG) stimulation test was also performed for certain 46, XY patients. Initial blood sample was collected for basal testosterone and dihydrotestosterone (DHT). Intramuscular injection of hCG (100 IU/kg) was given daily for 3 consecutive days. Blood sampling was repeated at 24 hours after last injection for interpretation. In normal prepubertal male, testosterone should increase at least 3-fold after hCG stimulation. A testosterone-to-DHT ratio after hCG stimulation exceeds 8.5 to 1 was considered as 5-alpha-reductase 2 deficiency[11,12]. Imaging such as ultrasonography and magnetic resonance imaging of abdomen and pelvis for determination of gonads, uterus, and/or vagina were performed at physician's discretion.

3. NGS library construction and sequencing

Genomic DNA was extracted from the peripheral blood samples of all included patients and their parents using the QIAsymphony DNA Midi Kit (Qiagen, Hilden, Germany). The quantification of genomic DNA was performed with the Qubit BR dsDNA kit (Invitrogen, Carlsbad, CA). The NGS library was prepared in accordance with the published commercial protocol from the Human Core Exome kit (Twist Bioscience, San Francisco, CA). Over 80% of human exomes have length of less than 200 bases[13] so we run 70-100 million paired-end reads per sample. The libraries derived from blood DNA were sequenced with Nova 6000sequencer (Illumina, San Diego, CA), achieving approximately 170 million reads per sample. Sequencing with a 151bp, dual-indexed, paired-end sequencing configuration was performed.

4. Raw data quality control, alignment mapping, and post-alignment

The obtained raw sequencing data of cDNA library was saved in FASTQ file format. Phred quality score was used to determine the accuracy of reads of the reference genome sequence[14]. The raw sequencing data was trimmed and filtered using Trimmomatic program. We used Burrows-Wheeler Aligner (BWA) and Bowtie for mapping and alignment of reads to human reference genome (hg 19) [15,16]. Post-alignment processing was performed using the Sequence Alignment/Map (SAM) format and SAMtools[17]. Alignments in the SAM format was converted into the binary alignment/map (BAM) format using the SAM tools. Duplicates were marked and indels were re-aligned. Base quality recalibration and metrics for depth were performed.

5. Data analysis and variant calling, annotation, and filtration

Variant calling was performed using the SAMtools and BCFtools for manipulation of variant call files (VCF) from which SNPs and indels were called[18,19]. The novelty of found variants were determined by comparison

with frequency of reported variants from 1000 Genome or gnomAD[20,21]. Variant Effect Predictor (VEP) and ANNOVAR were used for variant annotation. The effect of variants such as single nucleotide polymorphisms (SNPs), indels, copy number variations (CNVs), or structural variants were determined using VEP[22]. Functional annotation was performed with ANNOVAR for single nucleotide variants (SNVs) and indels according to functional impact and frequency of reports in the bioinformatic databases such as 1000 Genomes[23]. Variant filtration was performed by Variant Quality Score Recalibration using the in-house method. The variants which did not satisfy the following criteria were eliminated: 1) the variants showed an allele frequency < 1% in 1000 Genomes Project and gnomAD; 2) the variants were not found in our in-house database; 3) the variants were protein-altering variants; and 4) the variants had a high quality of reads (defined as read number > 20 or quality score [QS] > 30). Final candidate variants were selected by comparing against parents and prioritized based on the inheritance pattern.

6. Variant prioritization and assessment of pathogenic variants

The found variants were prioritized according to the American College of Medical Genetics and Genomics (ACMG) guideline[24]. Mode of inheritance, segregation, and frequency in general population were considered in terms of genetics. Gene function, pathway analysis, tissue expression, and disease models were also taken into account. The pathogenicity of found variants were assessed as stated in the ACMG guideline. The evidence of pathogenicity was classified as ‘very strong’ (including criteria PVS1), ‘strong’ (PS1-4), ‘moderate’ (PM1-6), and ‘supporting’ (PP1-5). The pathogenicity of variants found in our cohort were determined by the rules for combining criteria to classify variants. ‘Pathogenic’, ‘likely pathogenic’, and ‘variants of unknown significance (VOUS)’ based on the ACMG

guideline were selected. The workflow of the exonic variants and prioritization procedures are summarized in Figure 1.

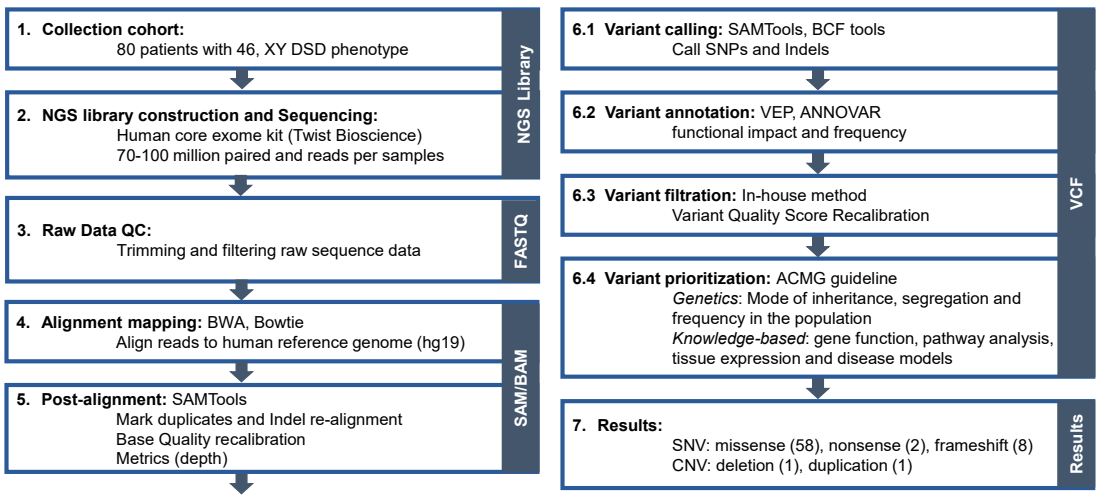

Figure 1. The workflow of the exonic variants and prioritization procedure

III. RESULTS

1. Patient characteristics

A total of 80 patients satisfied the inclusion criteria. Median age was 2 years (range: 1 month – 18 years). Eight patients were being raised as female at the study visit. The most commonly presenting phenotype was micropenis (N = 38), followed by hypospadias (N = 27). A wide spectrum of other phenotypes was observed: bifid scrotum (N = 2), gynecomastia (N = 1), delayed puberty (N = 1). Nearly a third of patients (30%, 24/80) presented with more than one phenotype. The external genital phenotypes of patients with genetic variations (N = 19) are illustrated in Figure 2.

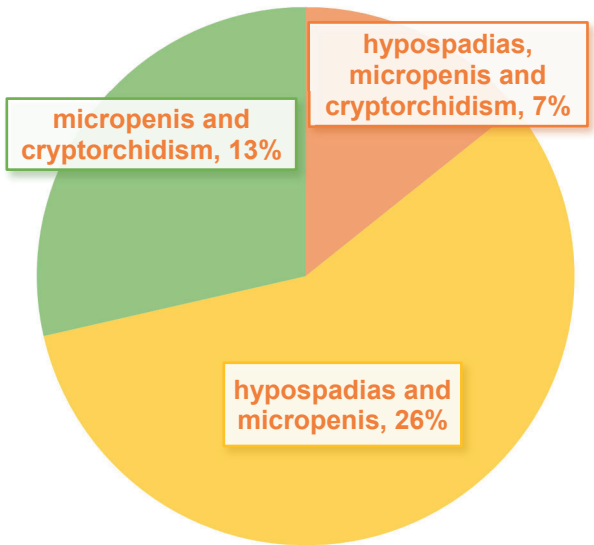

Figure 2. Proportion of 19 patients with gene variations in the phenotypic categories.

2. Detection of variant genes

Table 1 is a list of known genes of DSD previously used for targeted sequencing, of which this study used to filter the WES variants. The DSD genes, their role in pathophysiology, and reported phenotypes are shown. Among these genes, WES analysis of this study identified 20 variants in 11 genes from 19 patients. The basic statistics of WES data as gene variants identified in 19 patients are summarized in Table 2. Twelve pathogenic or likely pathogenic variants of 8 genes were detected in 12 patients. Of these variants, 4 (33.3%) were previously reported as pathogenic or likely pathogenic. Eight patients carried with a total of 8 VOUS detected in 7 genes. The most frequently affected genes were *NR5A1* (N = 4; 20%), *AR* (N = 2; 10%), and *FGFR1* (N = 2; 10%).

Table 1. List of targeted DSD genes in the WES

Gene	Alternative Name	Reported Associated Phenotype
Sex differentiation (e.g. steroid synthesis/receptors)		
AMH	*MIF, MIS*	PDMS
AR	*HUMARA, SMAX1, DHTR, AIS*	Complete AIS/partial AIS
ARX	*CT121, EIEE1, ISSX, MRX29, MRX32*	X-linked lissencephaly with ambiguous genitalia (XLAG)
AKR1C4	*CHDR, CDR, HAKRA, DD4*	46,XY DSD
ATRX	*XH2, XNP*	α-Thalassemia X-linked intellectual disability syndrome
CYP11A1	*P450SCC, CYP11A*	CAH
CYP17A1	*S17AH, P450C17*	17-α-hydroxylase-deficient CAH
FOXL2	*BPES1*	Blepharophimosis, ptosis, and epicanthus inversus
HSD17B3	*SDR12C2*	17-β hydroxysteroid dehydrogenase III deficiency
HSD3B2	*SDR11E2*	3-β-hydroxysteroid dehydrogenase-deficient CAH
LHCGR	*LHR, LCGR*	Leydig cell hypoplasia
MAMLD1	*CXorf6, F18, HYSP2*	Hypospadias
POR	*CPR, CYPOR, P450R*	Cytochrome P450 oxidoreductase deficiency
ROR2	*BDB1, BDB, NTRKR2*	Robinow syndrome
SRD5A2		Steroid 5-α-reductase deficiency
STAR	*STARD1*	Cholesterol desmolase-deficient CAH
ZFPM2	*FOG2, DIH3, SRXY9*	46,XY sex reversal, Diaphragmatic hernia 3, Tetralogy of Fallot
Sex determination (gonadal dysgenesis, testicular and ovotesticular DSD)		
BMP15	*GDF-9B, ODG2, POF4*	46,XY sex reversal; 46,XX premature ovarian failure
CBX2	*CDCA6, M33, SRXY5*	46,XY sex reversal
CYP11B1	*P450C11, FHI*	Adrenal hyperplasia, congenital

CYP26B1	*CYP26A2, P450RAI2, RHFCA*	46,XX sex reversal and campomelic dysplasia
DHH	*GDMN, GDXYM, HHG-3, SRXY7*	46,XY partial or complete gonadal dysgenesis
DMRT2	*DSXL-2*	46,XY gonadal dysgenesis
FSHR	*ODG1*	46,XY sex reversal; 46,XX premature ovarian failure
GATA4	*TOF,ASD2,VSD1,TACHD*	46,XY ambiguous genitalia
MAP3K1	*MAPKKK1, MEKK, MEKK 1, MEKK1, SRXY6*	46,XY sex reversal
NR5A1	*FTZF1, FTZ1, SF1, AD4BP, POF7, SRXY3, SPGF8*	46,XY sex reversal; 46,XX premature ovarian failure
NR0B1	*DAX1, AHC, AHX, NR0B1, SRXY2*	46,XY sex reversal
NR0B2	*SHP*	46,XY sex reversal
RSPO1	*CRISTIN3, RSPO*	46,XX sex reversal and palmoplantar hyperkeratosis
SOX3	*GHDX, MRGH, PHP, PHPX, SOXB*	46,XX sex reversal
SOX9	*CMD1, SRA1*	46,XX sex reversal and campomelic dysplasia
WNT4	*SERKAL, WNT-4*	46,XY DSD, 46,XY complete gonadal dysgenesis
WT1	*BMND16, INT1, OI15*	Wilms tumor-aniridia-genital anomalies-retardation syndrome
WWOX	*FOR, SCAR12, EIEE28*	46,XY gonadal dysgenesis
POF1B	*FLJ22792, POF2B*	46,XY sex reversal; 46,XX premature ovarian failure
SRY	*SRXX1, SRXY1, TDF, TDY*	46,XX testicular DSD and 46,XY ovarian DSD
ZFPM2	*DIH3, FOG2, SRXY9, ZC2HC11B, ZNF89B, hFOG-2*	46,XY sex reversal

Central causes of hypogonadism

ARL6	*BBS3, RP55*	Bardet-Biedl syndrome
BBS2	*BBS, RP74*	Bardet-Biedl syndrome
BBS5		Bardet-Biedl syndrome
BBS7	*BBS2L1*	Bardet-Biedl syndrome
BBS9	*B1, C18, D1, PTHB1*	Bardet-Biedl syndrome
BBS10	*C12orf58*	Bardet-Biedl syndrome
BBS12	*C4orf24*	Bardet-Biedl syndrome
CHD7	*CRG, HH5, IS3, KAL5*	Kallmann syndrome, normosmic IGD, CHARGE syndrome
GNRH1	*GNRH, GRH, LHRH, LNRH*	Isolated abnormality in GnRH secretion or response
GNRHR	*GNRHR1, GRHR, HH7, LHRHR, LRHR*	Isolated abnormality in GnRH secretion or response
HESX1	*ANF, CPHD5, RPX*	Combined pituitary hormone deficiency
HFE	*HFE1, HH, HLA-H, MVCD7, TFQTL2*	Hemochromatosis
LEP	*LEPD, OB, OBS*	Morbid obesity
MKKS	*BBS6, HMCS, KMS, MKS*	Bardet-Biedl syndrome/McKusick-Kaufman syndrome
PROKR2	*GPR73L1, GPR73b, GPRg2, HH3, KAL3*	IGD with anosmia (Kallmann syndrome) and normosmic IGD
PROP1	*CPHD2, PROP-1*	Combined pituitary hormone deficiency
TAC3	*HH10, LncZBTB39, NK3, NKB*	Isolated abnormality in GnRH secretion or response
TACR3	*HH11, NK-3R, NK3, NK3R, NKR*	Isolated abnormality in GnRH secretion or response
TRIM32	*BBS11, HT2A, LGMD2H*	Bardet-Biedl syndrome
TTC8	*BBS8, RP51*	Bardet-Biedl syndrome/retinitis pigmentosa, autosomal recessive
BBS1	*BBS2L2*	Bardet-Biedl syndrome

BBS4		Bardet-Biedl syndrome
FGFR1	*FLT2, OGD, KAL2, HH2, HRTFDS, ECCL*	Kallmann syndrome, normosmic IGD, and Pfeiffer syndrome
FGFR2	*K-SAM, CD332*	Apert syndrome
PCSK1	*PC1, PC3, NEC1, SPC3*	Morbid obesity
KAL1		IGD with anosmia (Kallmann syndrome)
LEPR	*CD295, LEP-R, LEPRD*	Morbid obesity
LHX3	*CPHD3, LIM3*	Combined pituitary hormone deficiency
FRAS1	*FRASRS1*	IGD with anosmia (Kallmann syndrome) and normosmic IGD
FGF8	*AIGF, FGF-8, HBGF-8, HH6, KAL6*	IGD with anosmia (Kallmann syndrome) and normosmic IGD
PROK2	*BV8, HH4, KAL4*	IGD with anosmia (Kallmann syndrome) and normosmic IGD
KISS1R	*AXOR12, CPPB1, GPR54, HH8, HOT7T175, KISS-1R*	Isolated abnormality in GnRH secretion or response

Abbreviations: CAH, congenital adrenal hyperplasia; IGD, isolated GnRH deficiency

Table 2. List of gene variants related to DSD in 19 patients

Pt No.	Gene	Variants	gnomAD Freq	Inheritance	Genotype	ACMG
1	NR5A1	NM_004959.4:c.938G>A (p.R313H)	.	AD, AR	hetero	Pathogenic
2	NR5A1	NM_004959.4:c.250C>T (p.R84C)	.	AD, AR	hetero	Likely pathogenic
3	NR5A1	NM_004959.4:c.260G>T (p.R87L)	.	AD, AR	hetero	Likely pathogenic

4	NR5A1	NM_004959.4:c.251G>T (p.R84L)	.	AD, AR	hetero	Likely pathogenic
5	SOX9	NM_000346.3:c.530G>A (p.R177Q)	.	AD	hetero	VOUS (PM2, PP2, PP3, PP4)
6	AR	NM_000044.4:c.1789G>A (p.A597T)	.	AD, XR	hemi	Pathogenic
7	AR	NM_000044.4:c.1540T>C (p.Y514H)	0	AD, XR	hemi	VOUS (PM2, PP2, PP3, PP4)
8	FGFR1	NM_023110.2:c.1898C>T (p.P633L)	.	AD	hetero	Likely pathogenic
9	FGFR1	NM_023110.2:c.550G>A (p.V184M)	0	AD	hetero	Likely pathogenic
10	MAMLD1	NM_005491.4:c.173G>C (p.G58A)	0	XR	hemi	VOUS (PM2, PP3, PP4)
11	MAMLD1	NM_005491.4:c.1497_1505del (p.499_502del)	0	XR	hemi	VOUS (PM2, PP4)
12	ZFPM2	NM_012082.3:c.960_961del (p.H320fs)	.	AD	hetero	Pathogenic
13	ZFPM2	NM_012082.3:c.535G>T (p.G179C)	0	AD	hetero	VOUS (PM2, PP3, PP4)
14	NR0B1	NM_000475:c.881T>C (p.L294P)	.	XR	hemi	Likely pathogenic
15	NR0B2	NM_021969.2:c.295_301del (p.G99fs)	.		hetero	Likely pathogenic

16	BMP15	NM_005448.2:c.787_788insATC (p.Leu262_Leu263insHis)	.		hemi	VOUS (PM2, PM4)
17	POF1B	NM_024921.3:c.884C>A (p.S295X)	0		hemi	Likely pathogenic
18	POF1B	NM_024921.3:c.1015C>T (p.R339W)	0		hemi	VOUS (PM2, PP3)
19	FRAS1	NM_025074.6:c.8933G>T (p.R2978L)	0	AR	compound hetero	VOUS (PM2, PP3, PP4)
19	FRAS1	NM_025074.6:c.6819T>A (p.Y2273X)	0	AR	compound hetero	Pathogenic

3. Diagnostic yield of WES

The final diagnostic yield was 15% (12/80) for all patients. Seven patients were identified with VOUS. The total variant detection rate, including pathogenic, likely pathogenic, and VOUS, was 24% (19/80).

4. Pathogenic and likely pathogenic variants identified by WES analysis

Gene mutations with pathogenic and likely pathogenic variants were identified in 12 patients. Causes of their mutations are presented in Table 2. Mutations for DSD were classified as pathogenic or likely pathogenic variants according to the ACMG guideline. Of these 12 patients, 8 (66.6%) had missense mutations, 2 (16.6%) had nonsense mutations, 1 (8.3%) had insertion or deletion mutations, and 1 (8.3%) had frameshift deletion.

Among the genes reported to be related to DSD in the literature, variants of seven known genes (*NR5A1, SOX9, AR, FGFR1, MAMLD1, ZFPM2, NR0B1*, and *NR0B2*) were identified in our cohort. Recurrent gene with highest

number of involved patients (N = 4) was *Nuclear Receptor Subfamily 5 Group A Member 1* (*NR5A1*). It is located in chromosome 9 with autosomal dominant pattern of disease when mutated. All 4 patients bearing mutations of *NR5A1* had missense mutations with novel variants. The variant in patient 1 was pathogenic while those found in patients 2, 3, and 4 were likely pathogenic according to the ACMG guideline. Patient 6 had a variant of the androgen receptor (*AR*) gene. It is located in X chromosome and is inherited in X-linked recessive manner. It was a missense mutation. He was found to have the variant c.1789G>A resulting in p.R569H and it was pathogenic according to the ACMG classification. Two patients had variants of *Fibroblast Growth Factor Receptor 1* (*FGFR1*) gene. It is an autosomal dominant gene located in chromosome 8. Both patients had missense mutation. Patient 8 had the variant c.2165C>T resulting in p.P633L. Both variants found in our cohorts were novel and likely pathogenic according to the ACMG guideline. Patients 12 and 13 were found to have variants of *Zinc Finger Protein, FOG2 Family Member 2* (*ZFPM2*). It is located in chromosome 8 and is autosomal dominant. The variant c.960_961del caused a frameshift deletion in p.H320fs. It was a novel variant classified as pathogenic.

Next group of genes with variants found in our cohort was *Nuclear Receptor Subfamily 0 Group B Member 1* (*NR0B1)* and *Nuclear Receptor Subfamily 0 Group B Member 2* (*NR0B2)*. *NR0B1* is located in X chromosome and is inherited recessively. *NR0B2* is located in chromosome 1 and is inherited in an autosomal dominant manner. Patient 14 with the variant c.881T>C resulting in p.L294P of *NR0B1* gene had a novel variant causing a missense mutation and was classified as likely pathogenic. The variant c.295_301del found in patient 15 resulting in p.G99fs caused a frameshift deletion in *NR0B2* gene. It was a novel variant classified as likely pathogenic. Patient 17 had the variant c.884C>A causing a nonsense

mutation resulting in p.S295X of *POF1B* gene. It was a novel variant classified as likely pathogenic. Patient 19 was found to have compound heterozygous variants of *FRAS1* gene. It is located in chromosome 4 and is autosomal recessive. The variant c.6819T>A resulting in p.Y2273X caused a nonsense mutation. It was a variant previously reported and was classified as pathogenic according to the ACMG guideline. The second variant was a VOUS.

5. Clinical characteristics of patients with pathogenic or likely pathogenic variants

Among 12 patients with mutations of pathogenic or likely pathogenic variants, only one patient was raised as a female. The external genital phenotypes of patients with genetic variations are illustrated in Figure 2. In case of *NR5A1* gene, Patient 1 with the variant c.938G>A resulting in p.R313H was a female patient who presented with clitomegaly and testicular inguinal hernia. She underwent clitoroplasty and orchiectomy at 23 months of age. Patient 2 who presented with hypospadias and micropenis had the variant c.250C>T resulting in p.R84C. Patient 3 presented with hypospadias at the penoscrotal area and had micro-penis and bifid scrotum. He had the variant c.260G>T resulting in p.R87L. Patient 4 with the variant c.251G>T resulting in p.R84L also presented with hypospadias, micropenis, and bifid scrotum. Except for patient 1 who were tested at 6 years of her age, 3 male patients were tested early at infancy within a year after birth.

Patient 6 with *AR* gene mutation was 10 years old who presented with hypospadias, cryptorchidism, and micro-penis. Patient 9 with *FGFR1* mutation who presented with perineal type hypospadias, bifid scrotum, and cryptorchidism had the variant c.550G>A resulting in p.V184M. Patient 12 with *ZFPM2* mutation was a teenager who presented with micro-penis. Patient 14 with *NR0B1* mutation presented with dermal pigmentation and

micro-penis at 14 years of age. Patient 15 with *NR0B2* mutation was an infant who presented with hypospadias at the scrotal area and micro-penis. Patient 17 with *POF1B* mutation was a teenager who presented with micro-penis and short stature. Patient 19 with *FRAS1* mutation and the patient's mother had identical compound heterozygous variants of the gene. Therefore, it is highly unlikely that the variants caused symptoms in Patient 19 considering the autosomal recessive inheritance of *FRAS1*.

6. Significant variants of unknown significance (VOUS)

A variant of *SRY-Box Transcription Factor 9* (*SOX9*) was found in patient 5. It is autosomal dominant and located in chromosome 17. Patient 5 was a girl who presented with ambiguous genitalia and underwent gonadectomy, vaginoplasty, and clitoroplasty at 3 years of age. She had a missense mutation with *de novo* variant c.530G>A resulting in p.R177Q. Patient 7 had a variant of the androgen receptor (*AR*) gene. It is located in X chromosome and is inherited in X-linked recessive manner. It was a missense mutation. Patient 7 presented with proximal penile hypospadias at infancy. He had the variant c.1540T>C resulting in p.Y514H and it was a novel variant in male with unknown significance. Variants of *MAMLD1* was found in two patients. It is located in X chromosome and is inherited in X-recessive manner. Patient 10, a teenager who presented with proximal penile hypospadias, had a missense mutation with the variant c.173G>C resulting in p.G33A, which had been previously reported in both male and female. It was classified as VOUS. Patient 11 presented with micro-penis at infancy and had a non-frameshift deletion with the variant c.1497_1505del resulting in p.474_477del. It was a novel variant classified as VOUS. Patient 13 with *ZFPM2* mutation was an infant who presented with hypospadias and micro-penis. The variant in this patient was a missense mutation (c.535G>T, p.G179C). It was a VOUS which had been previously reported. Patient 18

presented with hypospadias and micro-penis at 8 years of age. Patient 18 with *POF1B* mutation had the variant c.1015C>T resulting in p.R339W was a VOUS which had been previously reported. The second variant found in patient 19 with *FRAS1* mutation, c.8933G>T resulting in p.R2978L caused a missense mutation. This variant also had been previously reported and was classified as VOUS.

IV. DISCUSSION

Using WES, this study identified 12 novel variants from 8 genes. known to induce DSD: *NR5A1* (p.R84C, p.R84L, p.R87L and p.R313H), *SOX9* (p.R117Q), *AR* (p.R569H), *FGFR1* (p.V184M and p.P633L), *MAMLD1* (p.474-477 del), *ZFPM2* (p.H320fs), *NR0B1* (p.L294P), and *NR0B2* (p.G99fs). Five novel variants were also identified from three known genes with different phenotypes in 7 patients: *BMP15* (p.L263delinsHL), *POF1B* (p.R339W and p.S295X) and FRAS1 (p.R2978L and p.Y2273X). Out of 80 patients with clinically mild symptoms, 12 patients were identified with pathogenic or likely pathogenic variants for DSD. The diagnostic yield of WES was 15% in this study.

In the era of highly advanced genomic technology as nowadays, there are a plethora of gene panels and targeted sequencing developed for genetic diagnosis of a disease. Among numerous testing methods, we selected WES because of the complexity of symptoms and genetically heterogenous etiologies, both of which are main characteristics of DSD. Although major phenotypes have been identified in DSD, only a minority of patients conform to known phenotypes while most of the cases are heterogeneous. Moreover, the expressed phenotype changes dynamically throughout growth and development, which subsequently affects the results of analysis. Therefore, we considered the comprehensive approach achievable with WES was more appropriate for DSD compared to specific gene-targeting techniques.

With regards to the diagnostic yield of WES, the arithmetic rates are approximately 15-50% in previous reports[25,26]. The wide range of reported diagnostic yield is due to the heterogeneity of assessed cohorts and various stringency of diagnostic criteria among studies. In a Chinese data, the diagnostic yield was as low as 13%[25], while in a study on WES of neurodevelopmental patients the instant diagnostic yield was 47.5%[26]. The diagnostic yield of 15% in our cohort was within the range of previous reports.

Among the eight recurrent genes known to be associated with DSD in previous reports found in our cohort, *NR5A1* was the gene with four patients involved in our cohort. It encodes a protein which functions as a balancer between differentiation into testis or ovary by determining whether to stimulate *SOX9* and shift the pathway to development of testis or reinforce other pathways. With loss of function of *NR5A1*, there have been reports of failure of normal development or complete malformation of testis[27]. There are as much as nearly 200 reported mutations of *NR5A1* to date[28]. Among the four patients with mutations of *NR5A1*, three patients had variants located in the DNA-binding domain (DBD). Thus as a result of problems associated with transcription, it is likely that these variants have caused DSD in these patients. The variant p.R313H located in the ligand-binding domain (LBD) was found in a female patient, which may have caused DSD by poor ligand-binding and consequent impediment of downstream signal transduction for development of male phenotype.

A variant of *SOX9*, another gene with numerous reports of its relation to DSD, was found in a patient. Because it is autosomal dominant, it is likely that the variant of this gene may have caused DSD in the patient from our cohort. SOX9 functions as a chaperone by controlling in general the expression of Y chromosome with presence of sex-determining region Y (SRY) gene. It acts like a receptor which senses the level of testosterone and also like a transcription factor for differentiation of testis and development of skeleton or

other organs. It is known to cause campomelic dysplasia when affected and there are reports that complete sex reversal may be accompanied in those cases[29]. For the *SOX9* gene to function properly, the high mobility group box (HMG) domain is extremely important because it acts like an architectural transcription factor by binding DNA in a sequence-specific manner[30]. The variant p.R177Q found in patient 5 was located closely before the HMG domain which may act as a hinge to expose the HMG domain to function as a transcription factor.

AR is a gene which encodes a protein that acts as a receptor for androgen signal as well as a transcription factor. Testosterone acts through its metabolite, dihydrotestosterone (DHT), as AR binds DHT and transports it from cytoplasm into the nucleus. In the nucleus, AR acts as a transcription factor. The variants p.Y514H and p.R569H found in patients from our cohort are located in the so-called hot spots with numerous reported variants[31].

Another gene with variants in two of our patients was *FGFR1*. It encodes a receptor located in the cell surface which binds fibroblast growth factors that regulate cell proliferation, differentiation, and migration. Its role in DSD is known to be associated with failure of cell migration causing abnormal enlargement of one side along with poor development of its counterpart and ensuing malformation. Although it is a gene frequently tested for DSD, it is better known as a gene related to Kallmann syndrome which is often accompanied by hypogonadism[32]. Because FGFR1 is a tyrosine kinase, its major functional part is the tyrosine kinase domain (TKD). It activates or inactivates certain genes by phosphorylating tyrosine residues[33]. One of the patients had the variant p.P633L in the TKD, which may likely have brought functional loss of the gene. The variant found in the other patient was p.V184M, which is located within D2 of the immunoglobulin loops, and may have caused loss of function by bringing structural changes.

MAMLD1 is another gene with two patients found to have variants from our

cohort. It encodes a transcriptional co-activator which involves in differentiation of gonads, especially in the late androgen-dependent phase of development of external genitalia. It is reported as a causative gene for hypospadias and when this gene is affected, the growth of male external genitalia is halted rather than failing to differentiate[34]. Several nonsense mutations related to this gene have been reported in hypospadias. The patient with the variant p.G33A who presented with hypospadias had nonsense mutation, which is consistent with previous reports and we may suspect it as the pathogenic variant. The other patient with the p.474-477 non-frameshift deletion of this gene presented with micro-penis and cryptorchidism. Relating absence of a few amino acids as the cause of DSD may be a conjecture. However, the reported phenotypes of *MAMLD1* are extremely heterogeneous and there are previous reports of missense mutations or deletion associated with this gene in patients with micro-penis, cryptorchidism, and/or small testis. Thus, we considered current evidence sufficient for us to suspect the variant found to be associated with DSD[35].

Another gene, *ZFPM2*, was found to have variants in two patients from our cohort. It encodes a protein which is more famous as Friend of GATA 2 (FOG2), or "the second most friendly" FOG family member that is friends with GATA4. The patient with indel is suspected to have a frameshift deletion while the other patient had missense mutation, both of which presented with micro-penis. GATA and FOG2 form a complex to function on SOX9 and the domain for mutual recognition of these heterodimers is extremely important[36]. A problem with FOG2 recognition of GATA4 causes dysfunction of SOX9, which is known as the mechanism of DSD in affected patients[37].

Next group of genes with variants found in our cohort was *NR0B1* (Nuclear Receptor Subfamily 0 Group B Member 1) and *NR0B2* (Nuclear Receptor Subfamily 0 Group B Member 2). The patient with the variant p.L294P of *NR0B1* presented with dermal pigmentation and micro-penis. It encodes a

protein named DAX1 which functions as a repressor of steroidogenic factor-1(SF-1)-mediated activation of several genes engaged in steroidogenesis. The loss of inhibitory function of DAX1 has been demonstrated as responsible for pathogenesis of X-linked adrenal hypoplasia congenita[38]. The variant found in our cohort is located in the ligand-binding domain, which may have probably caused functional loss of the gene and the symptoms from our patient such as hyperpigmentation of skin is also in accordance with its function. *NR0B2* encodes SHP (small heterodimer partner) and is a gene seldom reported in DSD. It is autosomal dominant in contrast to *NR0B1* and a patient in our cohort had a frameshift deletion of *NR0B2*. DAX1 and SHP form a heterodimer to sense androgen and SHP acts on AR without involving Notch signaling[39]. Whether the frameshift deletion of *NR0B2* has caused DSD in our patient remains to be seen in additional functional studies.

Next, there were three known genes found in our cohort with different clinical manifestations from previous reports. Whether to include these patients in the diagnostic criteria is inconclusive due to incongruent phenotypes and warrants further study. Thus we did not include these patients in the diagnostic yield. A male patient with hypospadias had a variant of *BMP15*. Mutation of this gene is associated with ovarian failure or ovarian dysgenesis, which is far from male gonads and genitalia in terms of pathogenesis. BMP15 is known to be secreted from oocytes for maturation and development of ovarian follicles[40]. Another recurrent gene with variants in two patients of our cohort was *POF1B*. It is also known for ovarian failure and reported phenotypes are inconsistent those found in our patients such as hypospadias and micro-penis. The last known gene with discrepant phenotype was *FRAS1*. A patient in our cohort had compound heterozygous variants of this gene. Mutations of *FRAS1* cause disease in an autosomal recessive manner. Because it encodes a protein which regulates adhesion of epidermal-basement membrane and functions in organogenesis during development, Fraser syndrome caused by mutation of *FRAS1* is a

malformation involving multisystem such as craniofacial, genital, urinary, and respiratory abnormalities[41]. Although the patient with compound heterozygous variants of *FRAS1* presented with hypospadias and micro-penis, the phenotype of our patient is incompatible with multisystem involvement of the known syndrome. In addition, the patient's mother had identical variants without symptoms in the trio test.

To the best of our knowledge, this is the largest data of whole-exome sequencing in pediatric patients with DSD. Although imaging was not routinely obtained for definitive identification of gonadal dysgenesis, most of the patients included in this study presented with mild symptoms. In fact, diagnosis of patients with mild symptoms is more difficult for clinicians. Profound symptoms better rule out differentials of known genetic syndromes and specific gene panels may be used in those cases. However, mild symptoms such as in our cohort are more often encountered in the real-world practice. The value of this study is in exploring the potential of WES in diagnosis of DSD in patients with mild symptoms. Using WES, we have identified several known genes causative of DSD. Most patients with found variants had phenotypes concordant with previous reports. This study has demonstrated the additional role of WES in genetic diagnosis of DSD. The diagnostic yield of 15% is not low regarding the mild severity of DSD in our cohort.

Considering the rapid accumulation of newly reported variants on daily basis, the importance of reanalysis in NGS can never be underrated. Although this study successfully detected the previously known genes of DSD, further identification of novel candidate genes for DSD is necessary. In addition to reanalysis for novel genes, we are planning future studies for validation of recurrent genes with different phenotypes. Functional studies, either *in vitro* or *in vivo*, are warranted to investigate the molecular, biological, and biochemical functions of the found variants. Also, in regards with the dynamic characteristic of DSD with progression of growth, further follow-up of phenotypes as patients

grow up are also necessary.

V. CONCLUSION

In conclusion, WES is a useful tool for genetic diagnosis of a heterogeneous disease entity with widely variable phenotypes such as DSD. The diagnostic yield was 15% in our analysis of WES in 80 patients with mild symptoms. We demonstrated the additional role of WES in multidimensional approach for DSD.

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ABSTRACT(IN KOREAN)

전장엑솜시퀀싱을 이용한 한국인 46,XY 성분화이상 환자의 유전자 분석

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김용혁

목적 : 국내 성분화이상 환자에서 전장엑솜시퀀싱을 이용해서 원인 분석을 하고자함.

방법 : 모호생식기를 증상으로 세브란스 병원 소아청소년과를 방문하여 전장엑솜시퀀싱을 시행한 80명의 46,XY 성분화이상 환자를 대상으로 하였다. 대상군의 나이는 평균 2세 (1개월 – 18세) 였다.

결과 : 전장엑솜시퀀싱은 19명의 환자, 11개의 유전자에서 20개의 변이를 발견하였다. 80명의 환자 중 12명 (15%)에서 pathogenic 또는 likely pathogenic 유전자 변이가 확인되었다. 기존에 DSD를 유발한다고 알려진 8개의 유전자에서는 12개의 새로운 변이가 확인되었다: NR5A1 (p.R84C, p.R84L, p.R87L and p.R313H), SOX9 (p.R117Q), AR (p.R569H), FGFR1 (p.V184M and p.P633L), MAMLD1 (p.474-477 del), ZFPM2 (p.H320fs), NR0B1 (p.L294P), 그리고 NR0B2 (p.G99fs). 성분화이상이 아닌 다른 증상을 유발한다고 알려진 3개의 유전자에서 5개의 새로운 변이가 확인되었다: BMP15 (p.L263delinsHL), POF1B (p.R339W and p.S295X), FRAS1 (p.R2978L and p.Y2273X).

결론 : 국내에서 처음으로 46,XY 성분화이상 코호트를 대상으로 전장엑솜시퀀싱을 이용한 원인 분석을 수행하였다. 전장엑솜시퀀싱은 앞으로 성분화이상의 원인 유전자 변이를 찾아내는데 유용하게

활용될 수 있을 것이다.

핵심되는 말 : 46,XY 성분화이상, 전장엑솜시퀀싱, 유전자 검사