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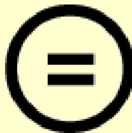

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Targeted next-generation sequencing panel-based mutation screening of Korean pediatric patients with idiopathic short stature and isolated growth hormone deficiency

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Targeted next-generation sequencing panel-based mutation screening of Korean pediatric patients with idiopathic short stature and isolated growth hormone deficiency

Directed by Professor Ho-seong Kim

Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University

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December 2021

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ABSTRACT

Targeted next-generation sequencing panel-based mutation screening of 144 Korean pediatric patients with idiopathic short stature and isolated growth hormone deficiency

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(Directed by Professor Ho-seong Kim)

Purpose: To investigate the distribution of mutation in genes causing short stature in Korean pediatric patients with idiopathic short stature and isolated growth hormone deficiency and to analyze clinical and molecular characteristics of patients with mutation identified through targeted next-generation sequencing panel.

Methods: A total of 144 patients aged between 5 and 19 years who were diagnosed with isolated growth hormone deficiency or idiopathic short stature and who visited the Department of Pediatrics at Severance Children's Hospital from January 2013 to December 2013 were selected as subjects of this study. Targeted next-generation sequencing panel for short stature was designed to include 96 genes.

Results: Identified heterozygous pathogenic or likely pathogenic genetic variants in 14 (10%) patients. Of the mutated genes, *PROKR2* (n = 3) is associated with gonadotropin-releasing hormone deficiency or hypopituitarism, while *FGFR1* (n = 1) and *NPR2* (n = 3) encode growth plate paracrine factors. *FBN1* (n = 1), *COL9A1* (n = 1), *MATN3* (n = 1), and *ACAN* (n = 3) regulate the cartilage extracellular matrix, while

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PTPN11 (n = 1) controls intracellular pathways. Six patients had IGHD, and eight patients had ISS.

Conclusions: We performed the genetic analysis of non-syndromic short stature for the first time in Korea. The patients with growth hormone deficiency and idiopathic short stature without dysmorphic features may have a gene defect in the growth plate and cartilage extracellular matrix. In cases of short stature cannot be diagnosed by the hormone test alone, a genetic test is required.

Key words: short stature, next-generation sequencing, idiopathic short stature, isolated growth deficiency

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Targeted next-generation sequencing panel-based mutation screening of 144 Korean pediatric patients with idiopathic short stature and isolated growth hormone deficiency

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(Directed by Professor Ho-seong Kim)

I. INTRODUCTION

Short stature, defined as individual height over two standard deviations (SDs) below the population ean, is a multifactorial condition caused by both genetic and environmental factors[1]. Non-genetic causative factors may include nutrition, chronic systemic disorders, as well as emotional or psychosocial deprivation. Nevertheless, hereditary factors account for 80% of human height determination and are thus regarded as the main causes of individual variation. However, 60–80% of these genetic factors are yet to be identified[2]. The etiology of short stature may differ with regard to the extent and type of growth retardation along with other clinical signs. Advances in diagnostic methods, especially during the second decade of the 21st century, have contributed to increasingly precise diagnosis[3]. Whole-exome sequencing has been introduced for the detection of gene variants as possible causes of congenital disorders, with a good diagnostic yield in well-selected patients[4]. The genetic defects that result in short stature can be classified as those affecting: 1) the growth hormone (GH), insulin-like growth factor 1 (IGF-1) axis; 2) the signaling of other hormones;
3) paracrine function of the growth plate; 4) cartilage extracellular matrix; 5) intracellular pathways; and 6) fundamental cellular processes, chromosomal abnormalities, copy-number variations, as well as imprinting disorders[5].

Most patients with short stature are non-syndromic, and the clinical signs

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associated with short stature are diverse, varying with age. Considering the large contribution of hereditary factors, genetic diagnostic tests can be used to predict disease prognosis and guide potential treatment for short stature as well as for future family planning. Thus, we developed such a test based on a panel of genes known to affect growth, which were described in a previous Dutch study[5]. Utilizing this panel, we sought to determine the genetic causes of isolated growth hormone deficiency (IGHD) and idiopathic short stature (ISS) in Korean patients presenting no dysmorphic features other than a short stature.

II. MATERIALS AND METHODS

1. Patient selection and study design

A total of 144 patients, aged between 5 and 19 years, who were previously diagnosed with IGHD or ISS and visited the Department of Pediatrics at Severance Children's Hospital from January 2013 to December 2013, were enrolled as subjects. ISS was defined as a height <3rd percentile without any other identifiable hormonal, skeletal, or systemic pathology. GHD was confirmed via GH stimulation tests using insulin (0.1 U/kg) and arginine (0.5 g/kg), with a peak GH response <7 ng/mL in both stimulation tests[6]. Exclusion criteria included: multiple pituitary hormone deficiencies; endocrine disorders such as hypothyroidism, hypoadrenalism, or hypogonadism; short stature due to Turner's syndrome, Prader–Willi syndrome, Noonan syndrome, Down's syndrome, or other chromosomal abnormalities; skeletal dysplasia; short stature due to being small for gestational age (SGA); short stature due to chronic renal failure or chronic inflammatory disease; or syndromes with growth disorders, such as Russell-Silver Syndrome and Seckel syndrome. Subject height, weight, and bone age (BA) were evaluated. Height was measured to the nearest 0.1 cm using a Harpenden Stadiometer. Weight was recorded to the nearest 0.1 kg. Growth parameters, including height and weight, were expressed as SDs and were calculated based on Korean children and adolescent growth standards[7].

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GH, and IGF-I levels were measured via chemiluminescence immunoassays using the Immulite 2000 Imunoassay System (Siemens Healthcare Diagnostics Inc., Germany). All assays have been previously validated for their limit of detection and quantification, precision, linearity, and recovery. The IGF-I SDS was calculated according to the Korean standard reference[8]. BA was assessed as per the Greulich-Pyle method by the same observer[9]. This study was approved by the Institutional Review Board of Severance Hospital (approval number:4-2012-0518). A written informed consent was obtained from all subjects or their parents before DNA isolation was performed. The study was conducted according to the principles described in the Declaration of Helsinki.

2. Panel design

First, we searched for diseases and genes associated with short stature using PubMed, Embase, and MEDLINE. Based on data from the Human Genome Mutation Database (HGMD), Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/omim), and an extensive literature review via PubMed, we extracted and optimized 96 disease-causing genes (Table 1).

Table 1. List of 96 genes in targeted NGS panel for short stature

Gene	Cytogenic location	Inheritance	Gene accession number	Disease association
ACAN	15q26.1	AD	NM_165800	Short stature and advanced bone age, with or without early-onset osteoarthritis and/or osteochondritis

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				dissecans
ADAMTS10	19p13.2	AR	NM_277600	Weill-Marchesani syndrome 1, recessive
ALMS1	2p13.1	AR	NM_203800	Alstrom syndrome
ANKRD11	16q24.3	AD	NM_148050	KBG syndrome
ARID1A	1p36.11	AD	NM_614607	Coffin-Siris syndrome 2
ARID1B	6q25.3	AD	NM_135900	Coffin-Siris syndrome 1
BMP2	20p12.3	AD	NM_617877	Short stature, facial dysmorphism, and skeletal anomalies with or without cardiac anomalies 1
BMPR1B	4q22.3	AD	NM_616849	Brachydactyly, type A1, D
BRAF	7q34	AD	NM_613706	Noonan syndrome
BTK	Xq22.1	XLR	NM_307200	Isolated growth hormone deficiency, type III, with

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				agammaglobulinemia
CHD7	8q12.2	AD	NM_612370	Hypogonadotropic hypogonadism 5 with or without anosmia
COL10A1	6q22.1	AD	NM_156500	Metaphyseal chondrodysplasia, Schmid type
COL2A1	12q13.11	AD	NM_200610	Achondrogenesis, type II or hypochondrogenesis
COL9A1	6q13	AD	NM_614135	Epiphyseal dysplasia, multiple, 6
COL9A3	20q13.33	AD	NM_600969	Epiphyseal dysplasia, multiple, 3, with or without myopathy
COMP	19p13.11	AD	NM_132400	Epiphyseal dysplasia, multiple, 1
CUL7	6p21.1	AR	NM_273750	3-M syndrome 1
FAM111A	11q12.1	AD	NM_602361	Gracile bone dysplasia
FBN1	15q21.1	AD	NM_614185	Geleophysic

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				dysplasia 2
FGD1	Xp11.22	XLR	NM_305400	Aarskog-Scott syndrome
FGF8	10q24.32	AD	NM_612702	Hypogonadotropic hypogonadism 6 with or without anosmia
FGFR1	8p11.23	AD	NM_101600	Pfeiffer syndrome
FGFR2	10q26.13	AD	NM_101600	Pfeiffer syndrome
FGFR3	4p16.3	AD	NM_100800	Achondroplasia
GDF5	20q11.22	AD	NM_615072	Brachydactyly, type A1, C
GH1	17q23.3	AD	NM_173100	Growth hormone deficiency, isolated, type II
GHR	5p13-p12	AD	NM_604271	Growth hormone insensitivity, partial
GHRHR	7p14.3	AR	NM_618157	Growth hormone deficiency, isolated, type IV
GHSR	3q26.31	AD,AR	NM_615925	Growth hormone

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				deficiency, isolated partial
GLI2	2q14.2	AD	NM_615849	Culler-Jones syndrome
GLI3	7p14.1	AD	NM_146510	Pallister-Hall syndrome
GNAS	20q13.32	AD	NM_174800	Albright hereditary osteodystrophy
HESX1	3p14.3	AD,AR	NM_182230	Growth hormone deficiency with pituitary anomalies
HMGA2	12q14.3	AD	NM_618908	Silver-Russell syndrome
HRAS	11p15.5	AD	NM_218040	Costello syndrome
IDUA	4p16.3	AR	NM_607014	Hurler syndrome
IFT172	2p23.3	AR	NM_615630	Short-rib thoracic dysplasia 10 with or without polydactyly
IGF1	12q23.2	AR	NM_608747	Growth retardation with deafness and mental retardation

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				due to IGF1 deficiency
IGF1R	12q23.2	AR	NM_608747	Growth retardation with deafness and mental retardation due to IGF1 deficiency
IGF2	11p15.5	AD	NM_616489	Silver-Russell syndrome
IGFALS	16p13.3	AR	NM_615961	Acid-labile subunit, deficiency of
IGSF1	Xq26.1	XLR	NM_300888	Hypothyroidism, central, and testicular enlargement
IHH	2q35	AD	NM_112500	Brachydactyly, type A1
IKBKB	8p11.21	AD	NM_18204	Immunodeficiency 15A
IL2RG	Xq13.1	XLR	NM_312863	Combined immunodeficiency, X-linked, moderate
KDM6A	Xp11.3	XLD	NM_300867	Kabuki syndrome

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KMT2D	12q13.12	AD	NM_147920	Kabuki syndrome
KRAS	12p12.1	AD	NM_613706	Noonan syndrome
LHX3	12p12.1	AD	NM_615278	Cardiofaciocutaneous syndrome
LHX4	1q25.2	AD	NM_262700	Pituitary hormone deficiency, combined,
MATN3	2p24.1	AD	NM_607078	Epiphyseal dysplasia, multiple
NRAS	1p13.2	AD	NM_613224	Noonan syndrome
NF1	17q11.2	AD	NM_162200	Neurofibromatosis, type 1
NKX2-1	14q13.3	AD	NM_610978	Choreoathetosis, hypothyroidism,
NPR2	9p13.3	AD	NM_616255	Short stature with nonspecific skeletal abnormalities
OBSL1	2q35	AR	NM_612921	3-M syndrome
OTX2	14q22.3	AR	NM_613986	Pituitary hormone deficiency,

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				combined,
PAPP-A2	17q23.2	AR	NM_615961	ALS deficiency
PIK3R1	5q13.1	AR	NM_269880	SHORT syndrome
PITX	4q25	AD	NM_180500	Axenfeld-Rieger syndrome, type 1
POC1A	3p21.2	AR	NM_614813	Short stature, onychodysplasia, facial dysmorphism, and hypotrichosis
POU1F1	3p11.2	AD,AR	NM_613038	Pituitary hormone deficiency, combined, 1
PRKAR1A	17q24.2	AD	NM_101800	Acrodysostosis 1, with or without hormone resistance
PROKR2	20p12.3	AD	NM_244200	Hypogonadotropic hypogonadism 3 with or without anosmia
PROP1	5q35.3	AR	NM_262600	Pituitary hormone deficiency, combined, 2

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PTHLH	12p11.22	AD	NM_613382	Brachydactyly, type E2
PTHR1	3p21.31	AD	NM_156400	Metaphyseal chondrodysplasia, Murk Jansen type
PTPN11	12q24.13	AD	NM_163950	Noonan syndrome 1
RAF1	3p25.2	AD	NM_611553	Noonan syndrome 5
RIT1	1q22	AD	NM_615355	Noonan syndrome 8
RNPC3	1p21.1	AR	NM_618160	Growth hormone deficiency, isolated, type V
ROR2	9q22.31	AD	NM_113000	Brachydactyly, type B1
RPS6KA3	Xp22.12	XLD	NM_303600	Coffin-Lowry syndrome
SEMA3E	7q21.11	AD	NM_214800	CHARGE syndrome
SHOX	Xp22.33		NM_300582	Short stature, idiopathic familial
SLC26A2	5q32	AR	NM_226900	Epiphyseal dysplasia, multiple, 4

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SLC5A5	19p13.11	AD	NM_274400	Thyroid dyshormonogenesis
SMARCA2	9p24.3	AD	NM_601358	Nicolaides-Baraitser syndrome
SMARCA4	19p13.2	AD	NM_614609	Coffin-Siris syndrome 4
SMARCAL1	2q35	AR	NM_242900	Schimke immunoosseous dysplasia
SMARCB1	22q11.23	AD	NM_614608	Coffin-Siris syndrome 3
SOS1	2p22.1	AD	NM_610733	Noonan syndrome 4
SOX2	3q26.33	AD	NM_206900	Optic nerve hypoplasia and abnormalities of the central nervous system
SOX3	Xq27.1	XL	NM_312000	Panhypopituitarism, X-linked
SOX9	17q24.3	AD	NM_114290	Campomelic dysplasia

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SPINK5	5q32	AR	NM_256500	Netherton syndrome
SPR	2p13.2	AD, AR	NM_612716	Dystonia, dopa-responsive, due to sepiapterin reductase deficiency
SRCAP	16p11.2	AD	NM_136140	Floating-Harbor syndrome
STAT3	17q21.2	AD	NM_615952	Multisystem, infantile-onset autoimmune disease
STAT5B	17q21.2	AD	NM_618985	Growth hormone insensitivity with immune dysregulation 2, autosomal dominant
TBCE	1q42.3	AR	NM_244460	Kenny–Caffey syndrome type 1
THRB	3p24.2	AD	NM_145650	Thyroid hormone resistance, selective pituitary
TRIM37	17q22	AR	NM_253250	Mulibrey nanism

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TSHR	14q31.1	AR	NM_275200	Hypothyroidism, congenital, nongoitrous, 1
WNT5A	3p14.3	AD	NM_180700	Robinow syndrome, autosomal dominant 1

Abbreviations: AD, autosomal dominant; AR, autosomal recessive

3. DNA preparation

A peripheral blood sample (3 mL) was collected from each patient in an EDTA tube. Genomic DNA was extracted from leukocytes using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of isolated DNA was validated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

4. Library preparation, target capture, and DNA sequencing

We constructed a DNA sequencing library using the Nextera Rapid Capture Enrichment protocol (TruSight One Sequencing Panel, FC-141-1007; Illumina, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, DNA in each sample was sheared to ~250-bp fragments, tagged, and purified according to fragment size. We subsequently performed repair, phosphorylation, and adenylation of 3´ ends and isolated pre-captured and amplified 300- to 500-bp fragments. We then performed targeted sequence capture according to the manufacturer's instructions (Illumina). DNA sequencing was performed on a MiSeq sequencer (Illumina), generating 100-bp paired-end reads. Image analysis and base calling were performed using the Illumina pipeline. The yield of each DNA sample averaged 2 GB of raw data with a 150-fold mean

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sequencing depth of targeted regions. Sequenced reads were mapped to the human reference genome (GRCh37). Sequencing alignment was performed using the Burrows–Wheeler Aligner software package.

5. Interpretation and analysis of detected variants

We analyzed NGS data and prioritized DNA variants according to clinical relevance based on the following parameters: 1) sequence quality; 2) allele frequency according to the Exome Aggregation Consortium (ExAC), dbSNP database, and Korean Reference Genome Databas (KRGDB;http://coda.nih.go.kr/); and 3) presence in HGMD, OMIM, dbSNP, or ClinVar. Real or possible damage to the variants was predicted using in silico prediction algorithms, including Polymorphism Phenotyping version 2 (PolyPhen-2) and Sorting Tolerant from Intolerant (SIFT; https://sift.bii.a-star.edu.sg/). After comprehensive analysis of all results, identified variants were classified into a five-tier system as pathogenic (P), likely pathogenic (LP), variant of unknown significance (VUS), likely benign, or benign as per the American College of Medical Genetics and Genomics (ACMG) guidelines[10]. According to inheritance patterns of the disease, results were considered positive if one to two P/LP variants in one disease-related gene were identified. The result was non-diagnostic if only a VUS or a single P/LP variant was detected in an autosomal recessive (AR) gene. We reported cases without any VUS or P/LP as negative results [11].

III. RESULTS

1. Characteristics of patients (n = 144)

A total of 144 patients were included in this study [median age: 8.39 years (range: 2.21–14 years)]. There were 84 (58%) males and 60 (42%) females. The average height standard deviation score (Ht-SDS) of patients was -2.85 ± 0.64 (range, -2.0 - -4.77). The average weight standard deviation score (Wt-SDS) of patients was -2.05 ± 1.19 (range, -0.02- -4.3). The average bone age was 7.08 ± 2.02 years. The bone age of most patients was less than their chronological age. From a total

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of 144 patients, 60 (42%) had IGHD, and 84 (58%) had ISS.

2. Detection of genetic variants

Targeted NGS analysis identified 402 variants in 72 genes, with every patient carrying at least one variant. On average, we detected 2.8 variants, with a maximum of seven per patient. We detected 20 P/LP variants of 11 genes in 19 patients. Of these variants, seven (35%) were previously reported as P/LP, whereas 13 (65%) were yet to be reported at the time of our investigation. In addition, all patients harbored one or more VUSs, with 377 VUSs detected in 71 genes. The most frequently affected genes were *ACAN* (n = 52; 36%), *KMT2D* (n = 17; 11.8%), and *ALMS1* (n = 16; 11.1%).

3. Diagnostic yield of NGS

The final diagnostic yield was 10% (14/144) for all patients. Nondiagnostic results were confirmed for 125 patients with VUSs only and for five patients carrying only one P/LP variant in the disease gene with an AR inheritance trait.

4. Clinical characteristics of patients with pathogenic and likely pathogenic variants

Clinical characteristics of patients with pathogenic and likely pathogenic variants are presented in Table 2. Mutations were identified in 14 of 144 patients. Nine of these 14 patients were males. The 14 subjects with confirmed mutations had an average age of 9.08 ± 2.01 years and an average Ht-SDS of -2.76 ± 2.07. Patients #5 and #6 as well as patients #11 and #12 were siblings. The average bone age was 8.24 ± 2.06 years. For most patients, the bone age appeared to be less than their chronological age, except for one patient carrying an ACAN mutation, chronological age, except for one patient carrying an ACAN mutation, which was associated with higher bone age.

The number of cases with height below 2SDs of either their mother's height or father's height was nine out of the 14 patients. Among the cases with confirmed mutations, six patients had IGHD, and eight patients had ISS. Patient #4 manifested clinical features of craniosynostosis. Despite a

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significative improvement in the IGF-I levels, only five patients presented a satisfactory response to GH treatment in the first year of treatment (gain greater than 0.7 SD).

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Table 2. Clinical characteristics of patients with pathogenic and likely pathogenic variants

Pt. No.	Gene	Sex	Age, y	Bone Age, y	Height SDS	Weight SDS	Mother Height SDS	Father Height SDS	Maximal GH level (ng/mL)	IGF-1 SDS	GH Dose (mg/kg/wks)	Height SDS (1yr after GH Tx)	IGF-1 SDS (1yr after GH Tx)
1	*PROKR2*	M	8.7	7.5	-2.47	-1.96	-2.35	-0.08	6.6	-0.66	0.22	-1.59	2.10
2	*PROKR2*	M	7.5	6.8	-2.45	-0.87	0.83	-0.24	3.1	-2.08	0.21	-2.08	-1.24
3	*PROKR2*	F	5.5	4.3	-2.13	-2.49	-0.54	-1.5	11.5	-0.32	0.20	-1.00	1.50
4	*FGFR1*	M	7.5	7.0	-2.01	-1.98	0.78	-2.14	3.5	-1.21	0.23	-1.77	-0.09
5	*NPR2*	M	9.7	7.4	-3.04	-1.87	-1.47	-4.61	12.6	-0.85	0.22	-2.72	0.49
6	*NPR2*	M	11.5	10.2	-2.92	-0.10	-1.47	-4.61	7.6	-1.17	0.22	-2.57	1.19
7	*NPR2*	M	11.5	9.5	-2.98	-1.02	-2.00	-0.45	5.0	-0.34	0.22	-2.30	3.41
8	*FBN1*	M	8.3	7	-2.18	-3.27	-0.34	1.68	8.9	-1.40	0.23	-0.52	0.62
9	*COL9A1*	M	13	11.5	-3.90	-2.27	-0.21	-0.26	9.6	-2.77	0.23	-3.56	-0.04
10	*MATN3*	F	9.4	9	-3.24	-2.51	-1.47	-1.01	5.9	-0.92	0.22	-1.71	1.4
11	*ACAN*	M	10	11	-3.00	-1.19	-0.21	-3.56	8.6	-0.83	0.2	-2.78	1.05

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12	*ACAN*	F	7.2	7	-2.73	-0.40	-0.21	-3.56	8.4	-0.50	0.23	-2.48	0.45
13	*ACAN*	F	7	7.4	-2.45	-0.65	-2.35	-2.74	6.6	-0.35	0.23	-2.13	2.06
14	*PTPN11*	M	10	10	-3.27	-2.70	-4.24	-2.74	16.8	-1.28	0.24	-2.07	0.45

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5. Pathogenic and likely pathogenic variants identified via TGNS

Gene mutations were detected in a total of 14 patients. Causes of their mutations are summarized in Table 3 [12-18]. According to guidelines presented by the American College of Medical Genetics (ACMG)[10], mutations for short stature were analyzed as pathogenic and likely pathogenic variants. Of these 14 patients, eight (57%) had missense mutations, four (28%) had stop-gain mutations, and two (15%) had frameshift mutations. *PROKR2* (n = 3) is associated with gonadotropin-releasing hormone deficiency or hypopituitarism, while *FGFR1* (n = 1) and *NPR2* (n = 3) encode paracrine factors in the growth plate. Four genes, including *FBN1* (n = 1), *COL9A1* (n = 1), *MATN3* (n = 1), and *ACAN* (n = 3), are involved in the regulation of cartilage extracellular matrix, while one gene *PTPN11* (n = 1) is implicated in the control of intracellular pathways. Patients #5 and #6 were siblings carrying an NPR2 mutation in the same location. Patients #11 and #12 were also siblings carrying *ACAN* mutations within the same site.

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Table 3. Pathogenic and likely pathogenic variants identified by targeted next-generation sequencing panel in 141 patients diagnosed with isolated growth hormone deficiency or idiopathic short stature

Pt. No.	Gene	Variants	Functional Annotation	Inheritance	Mutation on state	ACMG	Ex AC (All)	Ex AC (EAS)	Reference
	Causes of GHD								
1	*PROKR2*	NM_144773.2:c.533G>C (p.Trp178Ser)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0.01	[12,13]
2	*PROKR2*	NM_144773.2:c.533G>C (p.Trp178Ser)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0.01	[12,13]
3	*PROKR2*	NM_144773.2:c.533G>C (p.Trp178Ser)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0.01	[12,13]
	Examples of genetic defects affecting paracrine factors in the growth plate (FGF signaling)								

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4	*FGFR1*	NM_001174067.1:c.848C>G (p.Pro283Arg)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0	[14]
Examples of genetic defects affecting paracrine factors in the growth plate (CNP-NPR2 pathway)									
5	*NPR2*	NM_003995.3:c.895C>T (p.Arg299Ter)	Stop-gain	AD	heterozygous	Likely Pathogenic (I)	0	0	
6	*NPR2*	NM_003995.3:c.895C>T (p.Arg299Ter)	Stop-gain	AD	heterozygous	Likely Pathogenic (I)	0	0	
7	*NPR2*	NM_003995.3:c.1249C>G (p.Gln417Glu)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0	[15]
Examples of genetic defects affecting cartilage extracellular matrix.									
8	*FBN1*	NM_000138.4:c.7999G>A (p.Glu2667Lys)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0.01	[16]
9	*COL9A1*	NM_001851.4:c.2742delA (p.Phe915LeufsTer18)	Frameshift	AD	heterozygous	Likely Pathogenic	0	0	

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						(I)			
10	*MATN3*	NM_002381.4:c.209G>A (p.Arg70His)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0	[17]
11	*ACAN*	NM_013227.3:c.1551C>G (p.Tyr517Ter)	Stop-gain	AD	heterozygous	Likely Pathogenic (I)	0	0	
12	*ACAN*	NM_013227.3:c.1551C>G (p.Tyr517Ter)	Stop-gain	AD	heterozygous	Likely Pathogenic (I)	0	0	
13	*ACAN*	NM_013227.3:c.217delA (p.Ile73SerfsTer12)	Frameshift	AD	heterozygous	Likely Pathogenic (I)	0	0	
	Examples of genetic defects affecting intracellular pathways.								
14	*PTPN11*	NM_002834.3:c.846C>G (p.Ile282Met)	Missense	AD	heterozygous	Pathogenic (IIIb)	0	0	[19]

ACMG, American College of Medical Genetics and Genomics

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6. Significant variant of unknown significance

A trio test can be used to improve the pathologic scoring of the ACMG guidelines for 11 variants (Table 4). A de novo mutation was previously confirmed via a trio test, with an alteration of PM6 to PS2 representing a potential pathogenic variant. The same missense mutation was identified in three *ROR2* variants (c.626C > T, p.Ala209Va). However, as no trio test was performed in this study, it is difficult to establish its clinical significance.

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Table 4. Noteworthy variants of unknown significance

Pt. No.	Gene	Variants	Functional Annotation	Inheritance	Mutation on state	ACMG	Ex AC (All)	Ex AC (EAS)
16	*PROKR2*	NM_144773.2:c.308C>T (p.Ala103Val)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0.07
17	*ROR2*	NM_004560.3:c.1784T>C (p.Leu595Pro)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0
18	*ROR2*	NM_004560.3:c.626C>T (p.Ala209Val)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0.01
19	*LHX3*	NM_014564.3:c.701G>T (p.Arg234Leu)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0.04
20	*ACAN*	NM_013227.3:c.7124A>G (p.Gln2375Arg)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0

21	*ROR2*	NM_004560.3:c.626C>T (p.Ala209Val)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0.01
22	*MATN3*	NM_002381.4:c.659T>C (p.Val220Ala)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0
23	*NPR2*	NM_003995.3:c.1748T>C (p.Ile583Thr)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0
24	*NPR2*	NM_003995.3:c.2359C>T (p.Arg787Trp)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0
25	*NF1*	NM_001042492.2:c.3832A>T (p.Asn1278Tyr)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0
26	*ROR2*	NM_004560.3:c.626C>T (p.Ala209Val)	Missense	AD	heterozygous	PM2/PM6/PP3	0	0.01

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IV. DISCUSSION

Short stature is a common condition prompting referral to a pediatric endocrinologist. Various factors can influence the growth of children until they reach adult height, including heredity, hormones, and environmental factors. Several genes associated with short stature have been identified. Further, several diagnostic methods have been reported. Until now, studies primarily focused on syndromic short stature with various clinical features. However, ISS that is not associated with other condition. For this reason, we selected and analyzed 96 genes affecting the growth of patients with IGHD and ISS without dysmorphic features.

A total of 144 patients were tested using the 96-gene panel. Mutations were identified in a total of 14 (10%) patients. In a previous study, genetic mutations were identified in 33 out of 200 individuals with short stature, resulting in a diagnostic yield of 16.5%[19]. In the same study, a representative group of 200 children out of 565 subjects was selected, and ISS (67%) as well as syndromic short stature (37%) were investigated. According to Yang et al[20], 8 out of 91 patients with a short stature of unknown etiology had genetic mutations, resulting in a diagnostic yield of 9%. Their study involved 91 subjects with normal hormone secretion and ISS. The sequencing methods, number of patients, inclusion and exclusion selection criteria in the present study were different, but the diagnostic scope was similar.

Among the genetic mutations identified in 14 patients, one gene (*PROKR2*, n = 3) was associated with growth hormone deficiency in three patients, two genes (*FGFR1*, n = 1; and *NPR2*, n = 3) encoded paracrine factors affecting the growth plate in four cases, four genes (*FBN1*, n = 1; *COL9A1*, n = 1; *MATN3*, n = 1; and *ACAN*, n = 3) regulated the cartilage extracellular matrix in six cases, and one gene (*PTPN11*, n = 1) controlled intracellular pathways. All missense variants identified herein have been reported previously, and all mutations exhibited autosomal dominant inheritance.

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Previous studies have reported that *PROKR2* mutations are associated with gonadotropin-releasing hormone deficiency[12,13](p.Trp178Ser) and combined pituitary hormone deficiencies[21](p.Arg85Cys and p.Arg248Glu), suggesting that patients harboring these might exhibit various hypopituitarism features. In the present study, a *PROKR2* missense mutation was identified in one patient with ISS and two patients with IGHD. Three patients harboring this mutation did not exhibit defective puberty other than a short stature. However, because these patients were of an age before puberty onset, we could not completely rule out the possibility that hypogonadotropic hypogonadism may develop later.

Two genes mutated in four cases encode paracrine factors affecting the growth plate, namely *FGFR1* (n = 1) and *NPR2* (n = 3). One previously reported *FGFR1* missense variant was detected[14]. FGFR1 mutation is associated with craniosynostosis, which was also confirmed in our patient. Two *NPR2* mutations were found in siblings with stop-gain mutations. The third patient had presented a previously described *NRP2* missense mutation. *NPR2* encodes atrial natriuretic peptide receptor B (ANPRB), a regulator of skeletal growth. Biallelic loss-of-function mutations in *NPR2* result in acromesomelic dysplasia, while heterozygous mutations are associated with progressive short stature, exhibiting an increased loss of height with age[15,22]. Our three patients carried a heterozygous mutation and did have a short stature, yet without acromesomelic dysplasia.

Six cases harbored gene mutations affecting the cartilage extracellular matrix, including *FBN1* (n = 1), *COL9A1* (n = 1), *MATN3* (n = 1), and *ACAN* (n = 3) mutations. FBN1 mutations detected in the present study could be a cause of Marfan syndrome[16]. However, the patients with mutated FBN1 only exhibited ISS. According to a previous study by Le Goff and Cormier-Daire[23], *FBN1* causes acromelic dysplasias characterized by short stature, short hands and feet, stiff joints, and a hypermuscular build. These include Weill–Marchesani syndrome, geleophysic dysplasia, acromicric dysplasia, and Myhre Syndrome. Further, *FBN1* mutations are directly linked to a short stature phenotype and TGF-β

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signaling (Sakai et al., 2016). However, additional phenotypic and functional studies on *FBN1* mutations are necessary. *COL9A1* frameshift and *MATN3* missense mutations were identified in one patient with ISS and one patient with IGHD, respectively. Multiple epiphyseal dysplasia (*COL9A1*, *MATN3* mutations) occurring in early childhood is usually associated with pain in the hips or knees after exercise. Affected children complain of fatigue with long-distance walking[24]. Clinical manifestations of epiphyseal dysplasia were not observed in our patients at the time of this study. *ACAN* mutation was confirmed in three patients, including two siblings with stop-gain mutation and one carrying a frameshift mutation. *ACAN* mutation can accelerate bone mutation and cause early growth cessation. It is a common cause of familial short stature[25,26.] One patient with ISS in our study carrying this mutation showed short stature and advanced bone age. We identified a pathogenic variant in PTPN11 associated with Noonan syndrome in one patient with ISS. It was a confirmed missense variant. Facial features and typical cardiac malformation frequently trigger clinical suspicion and the diagnosis of Noonan syndrome. However, the patient analyzed in our study exhibited a mild phenotype that the patient analyzed in our study exhibited a mild phenotype that did not meet the criteria for clinical diagnosis[27-29].

With regard to the hormonal results of diagnosed patients, six patients had IGHD, and eight had ISS. Patients with IGHD carried mutations of *PROKR2*, *FGFR1*, *NPR2*, and *MATN3*. The *PROKR2* mutation has been reported to be associated with panhypopituitarism, while *FGFR1*, *NPR2*, and *MATN3* are associated with normal growth hormone secretion. This discrepancy between genotype and phenotype may arise from the arbitrary classification of GHD, without definite distinction between isolated GHD and ISS. Alternatively, functional abnormalities in GH secretion and unrelated genetic abnormalities may co-occur.

While the GH provocation test can be used for the diagnosis for GH deficiency, it cannot confirm the underlying causes of GH deficiency due to various

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limitations. Hormone tests lack precise cut-offs and have poor reproducibility, thus being useful only for determining the status of GH secretion, without insight into the specific causes of its dysregulation. In this study, several genetic mutations affecting the GH-IGF-I axis, paracrine factors in the growth plate, cartilage extracellular matrix, and intracellular matrix were identified in patients with short stature but without dysmorphic features. Freire et al[30] investigated 55 patients with ISS born SGA and identified genetic variants associated with growth plate development.

In a study conducted by Yang et al[20] involving 91 patients with short stature of unknown etiology, various mutations, such as *PTPN11*, *SOS1*, *ACAN*, *COL2A1*, *HOXD13*, and *COMP* causing syndromic short stature, were identified even in patients without definite deformity. These findings suggest that mild or atypical forms of syndromic short stature could induce growth disorders even in patients with short stature who lack dysmorphic features.

Therefore, gene mutations may be associated with atypical symptoms rather than consistent clinical features. In addition, if short stature is the sole clinical manifestation, the diagnosis could not be easily overlooked. The range of phenotypes should be expanded as genetic variations may not be associated with typical symptom manifestations.

Currently, there is no consensus on the genetic diagnosis of short stature. Further, the identification of rare monogenic causes remain imperative. Genetic tests are necessary in the cases such as severe GH deficiency, multiple pituitary hormone deficiency, unequivocal GH insensitivity, SGA without catch-up growth, additional congenital anomalies or dysmorphic features, evidence of skeletal dysplasia, associated intellectual disability, microcephaly, and a height below 3 SD[31]. Genetic testing can aid diagnosis, treatment, familial genetic counseling, decisions for growth hormone treatment, and the investigation of symptoms that are not apparent.

The limitations of the current study are related to the absence of trio testing

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and CNV analysis. These tests should be performed in the future to increase the diagnostic yield. Three identical *ROR2* variants were identified herein and should be further explored in future functional studies.

In summary, we performed genetic analyses of non-syndromic short stature Korean patients for the first time. Heterozygous pathogenic or likely pathogenic genetic variants were identified in 14 (10%) of 144 patients. Genetic testing may facilitate identification of the underlying causes of short stature, contributing to precise diagnosis and treatment decisions. Even in cases of short stature lacking dysmorphic features, a growth plate or a mild form of cartilage extracellular matrix gene defect may contribute to short stature. Taken together, TNGS panels can be used to identify the genetic etiology in some patients with IGHD and ISS.

V. CONCLUSION

We performed genetic analyses of non-syndromic short stature for the first time in Korea. Heterozygous pathogenic or likely pathogenic genetic variants were identified in 14(10%) of 144 patients. According to the results of this study, the hormone test and genetic test do not always coincide. Even in cases of short statures without the presence of dysmorphic features, a growth plate or a mild form of cartilage extracellular matrix gene defect may be the cause of the short stature. TNGS panel can be used to identify genetic etiology of some patients with isolated short stature.

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ABSTRACT (IN KOREAN)

특발성 저신장 및 단독 성장 호르몬 결핍증을 가진 144 명의 한국 소아 환자를 대상으로 한 차세대 염기 서열 분석 패널 기반 유전적 변이 스크리닝

<지도교수 김호성 >

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안정민

목적 : 국내 소아 특발성 저신장 및 단독 성장 호르몬 결핍 환자에서 저신장을 유발하는 유전자의 돌연변이 분포를 조사하고 표적 차세대 염기 서열 분석 패널을 통해 확인 된 돌연변이 환자의 임상 및 분자 적 특성을 분석 하고자 한다.
방법 : 2013 년 1 월부터 2013 년 12 월까지 세브란스 병원 소아청소년과를 방문하여 단독 성장 호르몬 결핍증 또는 특발성 저신장으로 진단받은 5 ~ 19 세 총 144 명의 환자를 대상으로 하였다. 표적 차세대 염기서열 분석 패널은 96 개의 유전자를 포함하도록 설계되었습니다.
결과 : 144 명의 환자 중 14 명 (10%)에서 이형 접합 pathogenic 또는 likely pathogenic 유전적 변이가 확인되었다. 이러한 유전적 변이는 이미 저신장과 관련이 있는 것으로 알려져 있디. 3명에게서 확인된 *PROKR2* 유전자 변이는 성장 호르몬 결핍과 관련이 있으며 성장판의 파라크린 인자에 영향을 미치는 *FGFR1* (n = 1)및 *NPR2* (n = 3) 유전자 변이가 확인되었다. 연골 세포 외 기질에 영향을 주는 유전자 *FBN1* (n = 1), *COL9A1* (n = 1), *MATN3* (n = 1) 및 *ACAN* (n = 3)의 변이는 6명에서 발견되었다. 세포 내 경로에 영향을 주는 *PTPN11* (n = 1)변이는 한 명이었다. 6 명의 환자는 단독 성장 호르몬 결핍이었고 8명의 환자는 특발성 저신장이었다.

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결론 : 국내 최초로 단독 저신장의 유전자 분석을 수행 하였다. 저신장은 호르몬 검사만으로는 진단 할 수 없으며, 단독 성장 호르몬 결핍 환자는 성장판과 연골 세포 외 기질에 유전자 결함이있을 수 있으므로 유전자 검사가 필요하다.

핵심되는 말 : 저신장, 차세대 염기 서열 분석 단독 성장 호르몬 결핍, 특발성 저신장