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# Pseudogenes: Nuances and Nuisances in Molecular Diagnostics

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Pseudogenes are genomic regions that contain gene-like sequences that have a high similarity to the known genes but are non-functional. They are categorized into processed, unprocessed, and unitary pseudogenes. Unprocessed pseudogenes generated by duplications can be problematic in sequencing approaches in molecular diagnostics. We discuss the risk of misdiagnosis when investigating genes with pseudogenes of high homology, and describe a method for identifying these small and annoying differences between parent genes and pseudogenes, including parent gene-specific assay design.

Key words: Pseudogene, Parent gene, Retrotransposition, Duplication

REVIEW ARTICLE

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## INTRODUCTION

Pseudogenes (Ψ) are genomic regions that contain gene-like sequences that have high similarity to the known genes (parent genes, i.e., their functional paralogs) but are non-functional. Therefore, pseudogenes have been considered as 'junk DNA' [1-6]. However, recent discoveries unveil this junk DNA's biological meanings, such as a regulatory role in cancer and developmental processes, and a genetic repository role to store and expand genetic information [7-14]. Explorations and annotations of pseudogenes can give an insight into their genetic evolution, diversification, and functionalization [15-17].

Pseudogenes can be introduced into our genome through several mechanisms such as spontaneous mutation that results in 'unitary pseudogene' [18], duplication that constitutes 'unprocessed pseudogene' [19], and, retrotransposition of processed mRNAs that produces 'processed pseudogene' [20]. Among them, unprocessed pseudogene maintaining its exonic and intronic structure could be problematic in molecular diagnostics because the genetic regions of pseudogenes and parent genes can be co-amplified, co-enriched, and co-aligned to the regions of interest. The mutation rate of pseudogenes is higher than that of the parent genes because they are not often under selection pressure. Therefore, mutations of pseudogenes can misguide the diagnostic approaches to the genetic disorders. Excluding out these false positive mutations with tiny differences ('nuances') in their flanking regions is challenging, and, sometimes, missed for geneticists, and, unfortunately, can be reported as a pathogenic variation to clinicians ('nuisances').

Here, we discuss the risk of misdiagnosis when investigating genes with pseudogene counterparts of high homology, and we describe the method of identifying these small and annoying differences between parent genes and pseudogenes, including parent gene-specific assay design.

## GENOMIC CONTEXT AND PSEUDOGENES

According to the current GENCODE release (version 41, 01. 2022.) that aims to annotate all genetic features at genome-wide level, human genome contains total 61,852 genes including 19,370 protein-coding genes (31%), 19,095 long non-coding RNA genes, 7,566 small non-coding RNA genes, and 14,736 pseudogenes (24%) [21]. Among pseudogenes, processed pseudogenes (10,662, 72%) comprise over two-thirds.

Pseudogenes could be identified by several characteristics such as the absence of introns, truncating mutations, and the absence of transcription. Although most pseudogenes are non-functioning, increasing numbers of pseudogenes have been demonstrated to play some biological roles.

Processed pseudogenes rarely have regulatory elements, thus lack transcriptional activities. *PGK2* (chr6:49,785,660-49,787,285) is a processed pseudogene of *phosphoglycerate kinase 1* (*PGK1*, chrX:77,910,739-78,129,295) via retrotransposition, but, it is expressed specifically in testis, encoding a functional phosphoglycerate kinase that catalyzes the reversible conversion of 1,3-bisphophoglycerate to 3-phophoglycerate, during spermatogenesis [22]. Some processed pseudogenes have intact open reading frames to be able to encode proteins, and play a biological role [23-26].

Unprocessed pseudogenes that arise via duplication preserve regulatory elements and intronic structures, but they are usually non-functional by disruption like truncation mutations. *NOTCH2* gene (chr1:119,911,553,120,100,779) has 4 identical paralogs (*NOTCH2NLA*, *NOTCH2NLB*, *NOTCH2NLC*, and *NOTCH2NLR*) via segmental duplications. *NOTCH2NLR* is likely to be non-functional, but other 3 *NOTCH2NL*s are expressed throughout corticogenesis [27]. Interestingly, *NOTCH2NLC* gene (chr1:149,390,621-149,471,833) is expressed significantly with age [28]. Recently, a heterozygous trinucleotide repeat expansion in the 5'-untranslated regions of the NOTCH2NLC gene was reported to be a causative mutation of neuronal intranuclear inclusion disease (NIID) [29, 30].

## MISLEADING AND CHALLENGING ASPECT OF SMALL DIFFERENCE IN DIAGNOSTICS

DNA sequencing by Sanger method or recent next-generation sequencing (NGS) technology is a crucial step for genetic diagnosis. This strategy can be hampered when there are homology regions with target regions of interest, mostly those are pseudogenes. Mutation data can be erroneous, especially when we analyze target genes with highly homologous unprocessed pseudogene counterparts because segmental duplications can be indistinguishable from their parent region if a laboratory is using short-read sequencing regardless of clinical or research settings.

Autosomal dominant polycystic kidney disease (ADPKD) is a common hereditary kidney disease caused by mutations in *PKD1* and *PKD2*. *PKD1* has 6 homologous pseudogenes (*PKD1P1-P6*) located between 13 and 16 Mb distant to *PKD1* via intrachromosomal duplication. Besides high GC contents and absence of hot spots in *PKD1* gene. high sequence homology (98-99%) of pseudogenes complicate the molecular diagnosis of ADPKD [31].

Gaucher disease is an autosomal recessive lysosomal storage disorder caused by mutations of the *GBA* gene, encoding the lysosomal enzyme acid β-glucosidase. Heterozygous *GBA* mutations are the main genetic risk factor for late-onset Parkinson disease. *GBA* also has a highly homologous (96-98%) pseudogene (*GBAP1*) located approximately 16 kb distant to *GBA* [32]. Thus, detecting a pathogenic variant in *GBA* is challenging with short-read sequencing because of alignment issues [33].

Filamin myopathy is a neuromuscular disorder caused by mutations of *FLNC* gene, encoding filamin C, muscle-specific filamin isoform cross-linking actin. *FLNC* has a highly homologous pseudogene (*pseFLNC*) located 53.6 kb distant to *FLNC*, which is 98% homologous to exons 46, 47 and 48 of the parent gene [34]. Genetic studies for *FLNC* had been erroneous for years until Odgerel et al. [35] discerned the misidentification of *FLNC* mutations and suggested an optimized strategy [36].

A recent study revealed recurrent GNAQ mutation encoding T96S in natural-killer/T cell lymphoma using NGS technologies [37]. However, mutations in this study was revealed to be misaligned to *GNAQ* instead of *GNAQP1*, a processed pseudogene of *GNAQ* [38]. When analyzing somatic mutations using fragmented DNAs, even processed pseudogenes can be problematic.

In the era of NGS technologies, pseudogenes can be still troublesome because short-read sequencing is more susceptible to mis-aligning of homologous sequences than conventional Sanger sequencing. If sequence reads of 150–250 bp containing a pseudogene-derived mutation are mapped to the parent gene, it will generate a false positive result. If sequence reads containing the parent gene-derived variant are mapped to the pseudogene, it will produce a false negative result.

## IDENTIFYING SMALL DIFFERENCE BETWEEN PARENT GENE AND PSEUDOGENE

When we devise a diagnostic assay to find a sequence variation in genetic regions of interest with highly homologous pseudogenes, we should confirm that the only desired genetic regions are amplified/enriched, aligned, and analyzed. The first step to do this is to identify small differences in sequence between parent gene and pseudogenes. The BLAST-Like Alignment Tool (BLAT) could be a good starting point to identify small differences [39]. BLAT is typically used to search similar sequence within the same species. Therefore, it is convenient for the identification of discrepant sites between two highly homologous genetic regions.

For example, Fig. 1 demonstrates how to identify the differences between the parent gene (*FLNC*) and pseudogene (*pseFLNC*) in the region of interest. We can annotate the discrepant sequence between parent gene and pseudogene upon the patient's sequencing reads to discriminate whether a significant variation is from parent gene or pseudogene.

## NEED FOR ORTHOGONAL METHODS: PARENT GENE-SPECIFIC ASSAY

When we cannot discriminate the significant variations from pseudogene-specific alterations in sequencing analysis, we need to find the other orthogonal methods, i.e., parent gene-specific assay. Mostly, we can adopt allele-specific amplification and subsequent Sanger sequencing that can achieve longer sequencing results than NGS. However, sometimes, we need additional separation or enrichment methods, such as cloning. We should manually design all primers and long-range PCR when confirming variants in regions with high homology and devising a new diagnostic assay for an already known gene with highly homologous pseudogenes. Fig. 2 shows the example of parent gene (*CEL*)-specific assay design and its application in Sanger sequencing. Using the parent gene-specific primer, we can exclude out pseudogene-derived sequence variations.

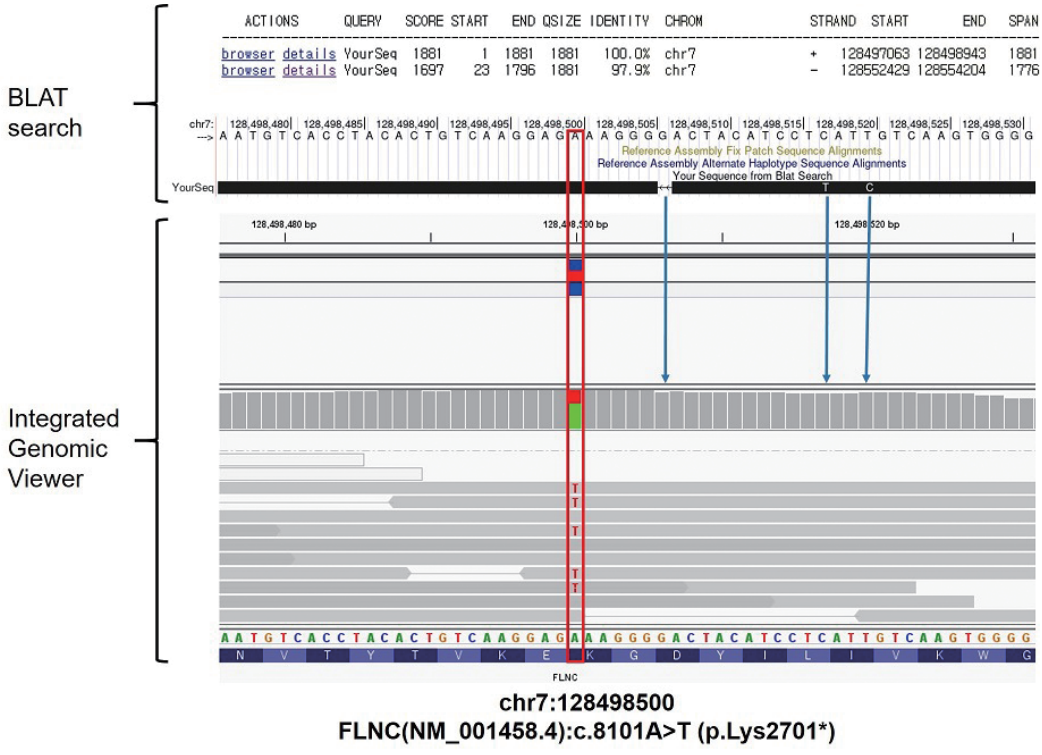



Fig. 1. Identifying differences in sequence between the parent gene and pseudogene using the BLAT search tool and genomic viewers from sequencing results. Through the BLAT search, similarity (97.9%) between *FLNC* exon 46–48 regions (chr7:128,497,063-128,498,943) and *pseFLNC* (chr7:128,552,429-128,554,204) was calculated. Patient's sequence reads are displayed in the Integrated Genomic Viewer (IGV). The red rectangle indicates a pathogenic variation of patient. Blue arrows show the discrepant sequences between *FLNC* and *pseFLNC*.

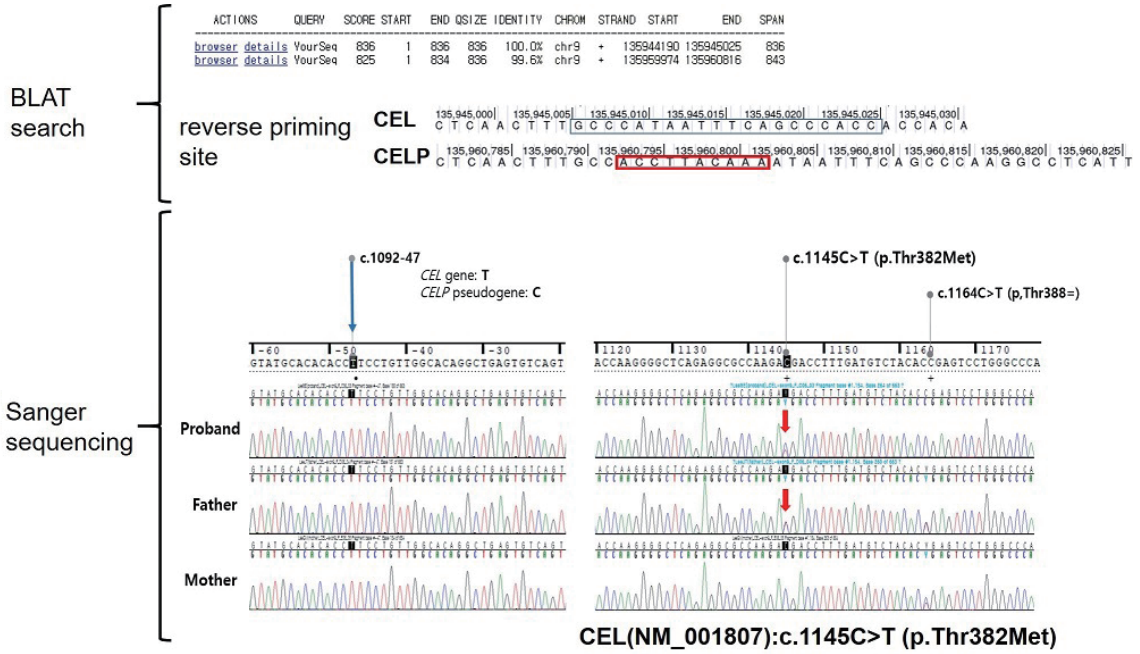



Fig. 2. Design of the parent gene-specific primer and its amplification. In the BLAT search, reverse primer binds parent gene (*CEL*)-specific region (blue rectangle). The red rectangle indicates discrepant sequences of pseudogene counterpart (*CELP*) with this reverse primer. In the Sanger reaction, the amplified product revealed parent gene-specific (c.1092-47T) results. Therefore, c.1145C>T variant comes from the parent gene.

## CONCLUSION

Even in the era of NGS, specific attention and considerations to discriminate pseudogenes from parent genes are needed. Investigators and clinicians should be aware of the possibility of false positive and/or false-negative results due to highly homologous pseudogenes. Laboratorians and researchers should be prepared for identifying small differences between parent gene- and pseudogene-derived sequences, and designing the parent gene-specific assays.

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## CONFLICTS OF INTEREST

None.

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# Klinefelter Syndrome: Review of the Literature

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Klinefelter's syndrome (KS) is a syndrome with extra X chromosome(s), in XY individuals, characterized by gynecomastia, small testes, and infertility. Additional X chromosomes can be present as variable karyotypic forms, including mosaicism (47,XXY/46,XY). The reported prevalence of KS ranges from one in 500 to one in 1,000 live males, but is probably underestimated. The classic phenotype is small, firm testes and infertility resulting from seminiferous tubule dysgenesis and androgen deficiency. The spectrum of KS includes tall stature with relatively long legs and arm span, decreased body hair, learning disabilities, behavioral problems, poor motor skills, and other important medical issues, such as metabolic syndrome, diabetes, autoimmune diseases, cardiovascular disease, certain neoplasia. The increased risk of certain medical problems in KS can be attributed to a direct effect of the extra X chromosome, the combined action of multiple genomic and epigenetic factors, or the hormonal imbalances. Typically, chromosome analysis is not ordered for adult patients with general medical conditions, except for suspected cases of hematologic and lymphoid disorders. Even though it was found during work-up for certain disorders in adult patient, most physicians do not suspect KS or consider its impact. Therefore, understanding the pathophysiology and variable manifestation in KS is necessary, and discussions with multidisciplinary teams will help to diagnose and treat males with KS.

Key words: Klinefelter syndrome, Pathophysiology, XXY

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## INTRODUCTION

Klinefelter's syndrome (KS) was first described 80 years ago [1]; 5 years after this initial publication, researchers found that men with KS have an excess number of X chromosomes [2]. KS is the most frequent chromosomal disorder in males, with an estimated prevalence of 1:500 to 1:1,000 live male births [3,4]. However, the actual prevalence is probably greater due to failure to identify, and a marked delay in diagnosing the syndrome may occur due to its variable phenotype.

Sometimes, the clone with extra-X chromosome has been found in bone marrow aspiration during work-up for hematologic or lymphoid disease in adult males. In these cases, additional tests should be performed to distinguish between malignant clones with extra X chromosome(s) and constitutional clones with KS. Most physicians do not suspect KS and do not consider its impact.

Here, I will review the prevalence of KS according to epidemiological studies, the pattern of karyotypes, KS genomics and epigenetics, and the risk for certain diseases in KS patients.

### Prevalence of KS

KS is the most common chromosomal disorder in males. The prevalence is estimated to be 85 to 223 per 100,000 liveborn males [95% confidence interval 121 to 188 per 100,000] [3-5], but this is probably an underestimate, as many cases go undiagnosed due to variable phenotype and severity.

According to the data from the Korean Statistical Information Service (KOSIS)

of the Ministry of Health and Welfare, the prevalence of KS in South Korea was extremely low in 2006: 3 per 100,000 live-birth males. (https://kosis.kr/statHtml/statHtml.do?orgId=117&tblId=DT_11776_N001) However, this KOSIS data is unreliable because the survey included only newborns. Danish data showed that 65% of prenatally diagnosed KS males are legally aborted, only small fraction are diagnosed in childhood, and a minority are diagnosed late in life. [3,4,6,7] This may have been a similar situation in Korea. Moreover, the prevalence differs among different racial groups within general population. In a study from the United States, the prevalence of KS in males with white ethnicity was 166 per 100,000 but 355 per 100,000 in males with Asian ethnicity [8].

### Karyotype variability in KS

The genotypes of KS are highly variable. Among them, the variation in phenotypes most likely depends on the number of extra X chromosomes, the number of abnormal cells, and their location in body tissues. The classic karyotypes seen at diagnosis of KS encompass 47,XXY or the various grades of mosaic karyotype 46,XY/47,XXY. Most KS males have the karyotype of 47,XXY. Approximately 15% of KS males are mosaic XY/XXY.

In comparison to XY/XXY, mosaicism with XX (46,XX/47,XXY) is rarely encountered. Mosaic XX/XXY is associated with a broad spectrum of phenotypes, from true hermaphroditism or genital ambiguity to (extremely rarely) normal female phenotype [9-13]. It is well known that both the phenotypic sex and the gonadal phenotype are influenced by the percentage and distribution of Y chromosome-bearing cells in the gonads, but not necessarily in the blood [14,15]. If the cell line in the gonads bearing a Y chromosome is predominant, the phenotype develops as male. This effect is based on the expression above a critical threshold level of the *SRY* gene (MIM*480000) in the developing urogenital ridge [16].

The reason for the rarity of XX/XXY cases is thought to be underestimated due to failure to detect the 46,XX cell line in the peripheral blood. Peripheral blood specimens are usually used for constitutional cytogenetic investigation. In prenatal study, mixed cells with 46,XX and 47,XXY in a culture dish might be considered contamination with the mother's cells. The presence of a 46,XX cell line has also been reported in cases of KS patients who have been mosaic for more than two different cell lines, XXY/XX/XY/X, XX/XY/XXY/XXXY, XX/XY/XXY/XXXY, XXY/XX/XY, XX/XY/XXY/XXYY, etc. [17,18]

In addition, there are rare cases including higher-grade X or Y chromosome aneuploidies (48,XXXY, 48,XXYY, 49,XXXYY) [19]. Previously, 48,XXYY has been reported as a variant of 47,XXY KS due to a shared physical and endocrinologic phenotype. However, it is now understood that 48,XXYY differs in its medical, neurodevelopmental, and behavioral characteristics [20]. Therefore, the karyotype of 48,XXYY was excluded in the classification of KS. Moreover, Tartaglia et al. [21] have emphasized that 48,XXYY, 48,XXXY and 49,XXXXY characterize a different syndrome from the classic 47,XXY KS, causing the development of more severe phenotypes. The most important reasons for exclusion are related to recognition of the associated medical problems that are more common in XXXY or XXXXY than in XXY, which often require additional evaluations, interventions, and treatments. Additionally, cognitive and behavioral problems in 48,XXYY, 48,XXXY, and 49,XXXXY tend to be more severe than in 47,XXY and require additional interventions and community supports [21].

### Genomics and epigenetics in KS

For decades now, phenotypic variability between individual and associated co-morbidities has been demonstrated. However, the effect of an additional X chromosome on the genome is still poorly understood. Several hypotheses concerning genetic mechanisms have been proposed and investigated.

#### Mosaicism between different tissue types

Mosaicism is considered one of the most important factors affecting clinical manifestation [22,23]. Usually, males with mosaic XY/XXY karyotype in blood samples present with a milder phenotype with reduced severity compared to non-mosaic KS males [24,25]. Moreover, evidence suggests that mosaicism may vary between tissue types and be more frequent than previously thought, based on conventional chromosome analysis using blood samples. Moreover, it is more frequent in cell types other than lymphocytes [26]. Garcia-Quevedo et al. [26] reported the highest degree of mosaicism in Sertoli cells (42.3±11.1%) and the lowest degree of mosaicism in lymphocytes (4.8±2.5%), whereas the degree of mosaicism was 21.9±10.9% in buccal mucosa.

#### Parental origin and inactivation of X chromosome

In 1989, it was proposed that imprinted genes on the X chromosome might be involved in the phenotypic variability of KS [27]. In addition, the hypothesis of skewed X chromosome inactivation has been suggested to explain the clinical heterogeneity of KS [28]. However, no imprinted genes on the

X chromosome have been identified, and other studies have not been able to find evidence of a parent-of-origin effect on phenotypes [23,29,30]. Moreover, evidence has not been found that there are different phenotypes between cases of skewed X chromosome and random inactivation in KS patients [31-33]. In order to establish this hypothesis, much larger unbiased studies are needed.

### Genetic variants of *AR* genes or autosomal genes

Usually, genetic variants can affect disease susceptibility or phenotype. The variants of *AR* gene (MIM*313700) on the X chromosome (Xq12) could be considered to influence phenotypic variabilities in KS patients [34]. The combined action of extra X chromosome(s) and autosomal or X-linked genetic risk variants might explain the increased risk of some disorders in KS patients [35]. To date, information to support these hypotheses is sparse. Additional, larger studies are needed to determine whether variants affect the phenotype of KS patients.

### Copy number variants on X chromosomes

According to a study, a proportion of KS males carrying X chromosome-linked copy number variants is significantly higher compared to controls [36]. Furthermore, some of the duplicated regions include genes in the pseudoautosomal region or genes escaping X-inactivation [36].

The X and Y chromosomes are comprised of two identical pseudoautosomal regions, PAR1 (24 genes) and PAR2 (4 genes). KS patients have an additional copy of the pseudoautosomal regions due to the extra X chromosome. A total of 15% of genes on the X chromosome, including all genes within PAR1, escape X-inactivation. Genes escaping X-inactivation are possible candidates affecting the phenotype due to a dosage effect. So far, only one pseudoautosomal gene, *SHOX* gene (MIM*312865), has been convincingly linked to the increased height of KS patients. Other genes in the pseudoautosomal region have been found to have different expression in KS, but the associations to phenotype have not been demonstrated [37,38].

### Other genetic studies

In one cell study, gain or loss of an X chromosome results in epigenetic instability by modifying the regulation process of transcriptional and translational pathways in the cells [39]. Epigenetic mechanisms, including DNA methylation, may be altered in KS, causing pervasive and global impact, and could therefore play a crucial role in KS phenotype [37,40]. To date, studies for transcriptome and epigenetics in KS patients indicate that the genetics behind KS may be more complex than previously assumed.

## Phenotype in KS

Many boys and men with KS do not realize that they have KS because the phenotype and severity of KS is highly variable. During the prenatal period, physicians may have the opportunity to diagnose and manage KS with prenatal genetic testing. Prepubertal features of KS are variable, and there are no symptoms that are present in all diagnosed cases. KS infants may have subtle dysmorphic signs and some degree of hypotonia [30,41]. Genital anomalies, such as micropenis, undescended testis, bifid scrotum, and hypospadias, are found more frequently in KS infants as compared to infants with a normal karyotype [42]. Congenital anomalies such as renal malformation and cardiac defect are rarely reported, and pathognomonic symptoms are not noted. According to some studies of testicular histology in KS males, the number of germ cells is already reduced at early age, whereas the number of Leydig cells appears to be normal [43-46].

Developmental delay is common in KS. Neurodevelopmental symptoms such as speech disturbance, adaptation problems, attention deficits, and social skill impairments can be found during the early pediatric period. As a result, KS males may be prone to learning difficulties [10,47,48].

The pubertal onset in KS boys occurs at the same time as in normal boys. Gynecomastia can develop due to the negative feedback of Luteinizing hormone elevation increasing estradiol levels. The signs of hypogonadism, such as poor muscular development and reduced facial and body hair, are noticeable during the adolescent period. Host et al. reported that most KS patients present signs of overt hypogonadism after the age of 25 [49].

In adulthood, KS males may have infertility, as diagnosed by azoospermia and oligospermia [31,50,51]. Previously, KS was considered to be a condition of absolute infertility. However, reports from many different centers revealed high sperm retrieval rates, excellent pregnancy rates, and healthy offspring with normal karyotype resulting from KS fathers after microdissection and testicular sperm extraction [51,52].

## Important medical conditions in KS

Apart from the apparent phenotypical features, KS is accompanied by a series of comorbidities and increased mortality risk [31]. Conditions leading to hospitalization include dyslip-

**Table 1.** Adult cases confirmed as Klinefelter syndrome in the work-up for hematologic disorder in Korea

| Case | Age[1] | Karyotype | FISH,X/Y[2] | Hematologic disease | Etc. |
|---|---|---|---|---|---|
| 1 | 59 | 47,XXYc,add (11) (q13) [20] | 2G1R | Bleeding tendency | Infertility, Follow-up<br>*No physical signs of KS |
| 2 | 63 | 47,XXYc | 2G1R | Aplastic anemia,<br>T-cell lymphoma | allo-SCT after 1 yr 9 mo of Dx; Death after 2 yr 7 mo of Dx |

[1]Age at confirmation of KS; [2]FISH on Buccal epithelial cells.

idemia, obesity, metabolic syndrome, prothrombotic tendency, autoimmune disease, osteoporosis, and cancers [51,53].

Serum testosterone levels are independently associated with insulin resistance, which suggests that low testosterone might be responsible for type 2 diabetes and the five-fold increase in metabolic syndrome has been observed in KS males [54,55]. We therefore could expect high efficacy for testosterone replacement therapy (TRT) on glucose metabolism in KS; however, TRT has not been shown to improve metabolic syndrome or diabetes in KS patients, compared to other types of hypogonadism [55,56]. This indicates that there are more complex mechanisms associated with KS and glucose metabolism.

In vascular and cardiac disease, KS males exhibit increased risk of venous thromboembolism: hazard ratio (HR) 5.29 for thrombophlebitis and venous thrombosis, HR 3.60 for pulmonary embolism, and HR 1.71 for ischemic heart disease [42,57,58].

A retrospective study from England has demonstrated a significant increased risk in KS relative to controls regarding Sjogren's syndrome (risk ratio (RR) 19.3), systemic lupus erythematosus (RR 18.1), Addison's disease (RR 11.7), diabetes mellitus type 1 (RR 6.1), multiple sclerosis (RR 4.3), rheumatoid arthritis (RR 3.3), and acquired hypothyroidism (RR 2.7) [59]. It was reported that the human X chromosome contains the largest number of immune-related genes, and around 10% of all microRNAs, from the whole human genome. Some microRNAs on the X chromosome have important immune-related functions.

KS is also an important risk factor for osteoporosis and osteopenia [31,60]. Bone disease in KS was eight-fold higher than the incidence in non-KS [61]. The etiology is multifactorial, with testosterone deficiency as the primary cause. Testosterone deficiency can lead to lower bone formation and higher bone resorption. TRT has been shown to improve bone mineral density (BMD) on some levels, but vitamin D repletion has been demonstrated to be superior to TRT for improving BMD [61-63].

In terms of the incidence of cancers, the difference of overall cancer risk is no significant between KS and the general male population. However, certain cancers, such as breast cancer and germ cell tumors, especially extragonadal tumors, occur more frequently in KS males than in the general population [64]. Compared with non-KS, the incidence of breast cancer is increased 4 to 30-fold, and the mean onset age of breast cancer is earlier (58 years vs. 67 years) in KS. This supports the idea that KS is the strongest independent risk factor for breast cancer in males [65-67]. In extragonadal germ cell tumors, higher prevalence is observed in KS with presentation at a younger age than in normal males [68]. On the other hand, the prevalence of prostate cancer and its associated mortality have been reported to be lower in KS than in normal-karyotype men [69,70].

Published studies regarding the risk of hematological and lymphoid malignancies in KS present conflicting evidence. Some publications suggest an increased risk of leukemia development in KS, whereas others have determined that there is only a chance association [69,71,72]. A Swedish registry study reported a notable increase in hematologic malignancies (standardized incidence ratio (SIR) 2.72) such as non-Hodgkin lymphoma (SIR 3.02) and acute leukemias (SIR 3.62) [69]. The underlying mechanisms for the increased incidence of these malignancies in KS are not clear and need to be investigated.

In my experience as a cytogeneticist, I have analyzed approximately 3,200 karyotypes of bone marrow specimens (1,800 cases of male patients) during a 10-year-period, 2011-2020. I met four adult men suspected to have KS; they were involved in the work-up for hematologic disorder. Patients suspected of having KS should undergo karyotyping on peripheral blood lymphocytes in remission state or fluorescence in situ hybridization (FISH) on buccal epithelial cells. Using this method, I was able to confirm KS in two patients aged 60-70s (Table 1). The others were not performed further evaluation.

## CONCLUSION

KS is the most common sex chromosome disorder in males. Despite increasing knowledge of the natural history of KS, di-

agnosis often does not occur throughout the lifespan. Earlier diagnosis of KS may provide greater opportunities for earlier intervention and effective treatment. Once the diagnosis of KS has been established, careful management and appropriate treatment are possible with multidisciplinary teams, including physicians. To help these patients early and achieve proper diagnosis, the deeper understanding of the pathophysiology of KS will be helpful.

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## CONFLICTS OF INTEREST

None.

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# Microarray Analysis of Extracranial Arteriovenous Malformation Endothelial Cells

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**Background:** Arteriovenous malformations (AVMs) are rare diseases comprising abnormally dilated arteries and veins with an absence of a capillary network. Since these diseases are intractable after diagnosis, various treatment strategies have been examined, with continuous efforts to identify target genes. Here, we report relevant new target genes selected via gene microarray. **Methods:** Endothelial cells were isolated from samples collected from three patients with AVM and three healthy individuals, followed by microarray analysis. Additionally, quantitative PCR was performed to select genes highly relevant to AVM. **Results:** In the vascular endothelial cells derived from the tissues of patients with AVM, the expression of *ANGPT1, ANGPT2, DLL4, IL6, NRG1, TGFBR1,* and *VEGFA* was typically higher compared to those derived from normal tissues. **Conclusion:** Seven candidate genes were selected to analyze the pathophysiological mechanism of AVM. These results may aid in future directions of diagnosis and treatment.

Key words: Arteriovenous malformations, Microarray, Vascular malformation

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## INTRODUCTION

Among vascular malformations, arteriovenous malformation (AVM) is one of the rare diseases in which the high-pressure blood flow of the artery is directly connected to the draining vein without an intervening capillary network [1-3]. Currently, it is difficult to treat extracranial AVM after diagnosis. To understand the molecular process underlying this intractable rare disease, several studies have examined the basic vascular architecture and differences in the expression of AVM-related biomolecules or angiogenesis-related genes [1,2,4-14]. Expressed genes are being continuously analyzed using the DNA microarray technique based on the results of other studies. Furthermore, treatment strategies are being developed via functional analysis of differentially expressed target genes [9-11]. Accordingly, we report the overexpression of new target genes in extracranial AVM using microarray.

## METHODS

### Sample collection and clinical information

This study was approved by the Institutional Review Board of Kyungpook Na-

tional University Hospital (approval number: KNUH 2020-03-078-001) and was performed in accordance with the Helsinki Declaration. Sampling was performed after obtaining consent from three patients with extracranial AVM and three healthy individuals. Using paraffin-embedded tissue of arteriovenous vascular malformation, hematoxylin and eosin (H&E), trichrome, elastin, and immunocytochemical staining methods were performed to observe histological morphology.

### Isolation of vascular endothelial cells from samples

In the case of vascular endothelial cells, after obtaining patient consent, vascular endothelial tissues to be discarded were obtained after resection. We then cut them with scissors after sterilization and placed the tissue sections in Dispase II (17105-041, Gibco), followed by incubation at 4°C for 12 to 24 hours. After soaking the tissue, the epidermis and dermis were separated. Thereafter, the dermal layer was collected in 10 ml of HEPES BSS (14170-112, Invitrogen) and submerged, followed by the removal of the supernatant. A total of 5 mL of collagenase Type I was added to the supernatant followed by incubation in a water bath. Subsequently, 10 mL of EMB-2 media (cc-3156, Lonza) was added and the contents were passed through a nylon filter with a pore size of 70 μm. After separating using a centrifuge (2,000 rpm, 3-5 minutes), cells were grown in the EMB-2 media (cc-3156, Lonza). During subculture, vascular endothelial cells were isolated while proliferating the cells using the CD31 Microbead kit (midiMacs starting kit set, 130-042-501).

### Quantitative real-time RT-PCR

Molecular biological analysis was performed to confirm the isolation of the vascular endothelial cells. First, RT-PCR found that *CD31*, a marker of vascular endothelial cells, was strongly expressed in the isolated vestibular endothelial cells when compared with that in the control group. Furthermore, pure vascular endothelial cells were isolated, as demonstrated by the morphology in the cell images. Endothelial cells were extracted from AVM region and normal vessels, and microarray hybridization was performed. Genes related to AVM, such as those participating in angiogenesis and cell proliferation with large fold changes were selected, resulting in a selection of 12 genes with high relevance. After the isolation and culture of vascular endothelial cells derived from patients with AVM patients, PCR amplification products were monitored in real-time. To this end, real-time PCR was performed because accurate quantification of exponential amplification ability is possible, contamination risk is low, and electrophoresis is not required, enabling a rapid and simple interpretation, to verify the expression pattern of target genes that are differently expressed in vascular endothelial cells. A PCR mix was prepared with 7 μl of SYBR Green Master mix (ABI 4367659), 1 μl each of forward primer (10 μM) and reverse primer (10 μM), and 1 μl of 300 ng/μl of cDNA, which was applied to PCR using a real-time PCR ABI 7500. The results were analyzed using Ct values and standard curves.

### Microarray hybridization and data analysis

To identify rarely expressed genes and to select target genes, endothelial cells with proliferative arteriovenous malformation were cultured, followed by RNA isolation. Probe synthesis, hybridization, detection, and scanning of total RNA (5 μg) were performed according to the manufacturer's instructions (Affymetrix; USA). First, recombinant complementary DNA (cDNA) was synthesized using total RNA. After the cleaning process, double-stranded recombinant cDNA was synthesized. In vitro transcription was performed using the recombinant cDNA as a template and biotin-bound CTP and UTP. After the cleaning process, 10–15 mg of biotin-bound recombinant RNA was fragmented using a fragmentation buffer (Affymetrix, USA). Thereafter, the recombinant RNA was hybridized to the HuGene-1-0-st chip using the standard experimental method (Affymetrix), and then the unbound probe was washed using the GeneChip Fluidics station 450. After washing, the chip was stained with streptavidin-phycoerythrin, and the signal strength was analyzed using the GeneChip scanner 3,000 of Affymetrix and GCOS software to obtain data. Gene expression patterns (Affymetrix microarray data) were analyzed using the Expression console version 1.1 (Affymetrix, USA). Absolute analysis metrics were determined using the Affymetrix Expression Console version 1.1. Change, change p-value, and signal log ratio (fold change) values were obtained by comparison between experimental groups. To reduce the deviation of each value, the number of each experiment was increased to average the fluorescence intensity. RMA-stretch was used for normalization. From the scanning results, genes with up-regulated or down-regulated expression by 1.68-fold or higher were selected. Additional analysis was performed using the upstream regulator analysis in the ingenuity pathway analysis (Ingenuity Systems, USA) methods.

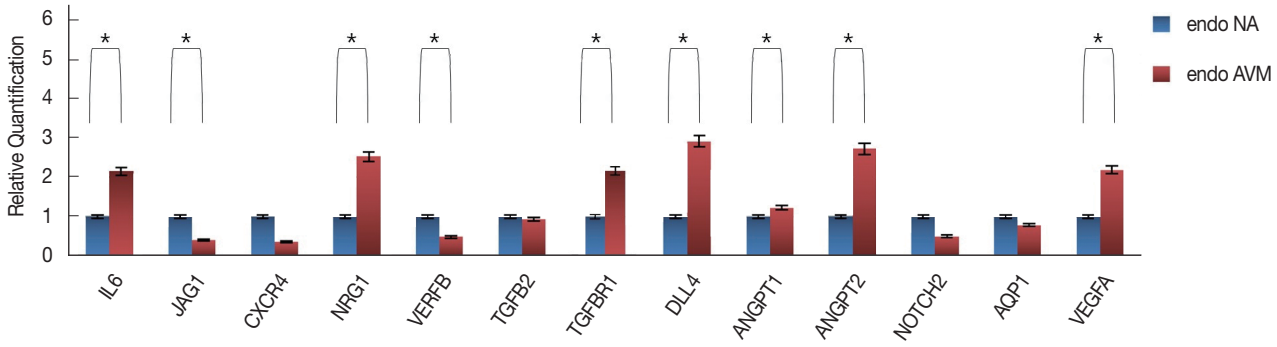



Fig. 1. Real-time PCR (quantitative PCR) analysis of arteriovenous malformation vascular endothelial cells. Endo; endothelial cells, NA; normal artery, AVM; arteriovenous malformation.

Table 1. Seven Genes Selected in the Microarray Data Obtained After Analysis of Arteriovenous Malformation Endothelial Cells and Normal Endothelial Cells

| | Symbol | Fold change | Gene |
|---|---|---|---|
| 7982854 | DLL4 | 0.207 | Delta-like 4 |
| 7955290 | ANGPT1 | 2.738 | Angiopoietin 1 |
| 8145766 | NRG1 | 0.533 | Neuregulin-1 |
| 8119898 | VEGFA | 5.301 | vascular endothelial growing factor-a |
| 8112139 | IL6 | 9.608 | Interleukin-6 |
| 8149071 | ANGPT2 | 0.184 | Angiopoietin 2 |
| 8156826 | TGFBR1 | 1.248 | Transforming growth factor β-receptor 1 |

## RESULTS

### Microarray data analysis of endothelial cells with AVM

The expression patterns of vascular endothelial cells derived from normal tissues and vascular endothelial cells obtained from tissues of patients with AVM patient tissues analyzed by real-time PCR showed increased expression of *ANGPT1*, *ANGPT2*, *DLL4*, *IL6*, *NRG1*, *TGFBR1*, and *VEGFA* in vascular endothelial cells of patients with AVM compared to those in normal tissue. Similar or decreased expression of *AQP1*, *CXCR4*, *JAG1*, *NOTCH2*, *TGFB2*, and *VEGFB* genes was observed in the vascular endothelial cells of patients with AVM compared to that in normal tissues. (Table 1, Fig. 1).

## DISCUSSION

AVM is one of the rare congenital diseases with common symptoms such as cutaneous stains, local warmth, palpable thrill, and bruit. In later stages, progression to ischemic conditions or indolent ulceration, which can lead to intractable pain, intermittent bleeding, and even congestive heart failure. Treatment typically involves blocking the feeding vessel via embolization. Sclerotherapy, surgical resection, or an appropriate combination of these treatments are also used. In particular, for surgical resection, the risk is low when performed after embolization and 24 to 72 hours before surgery. The success rate is the highest in the case of well-localized AVMs in stages I or II. The best way to determine the resection margin is to clinically examine the bleeding pattern of the wound edges. However, it has a high recurrence rate and may require repeated several surgical treatments. Hence, etiological target genes that can be used to simultaneously diagnose and treat the disease are being continuously examined. In this study, we aimed to report new genes related to AVM by analyzing target genes through microarray. Vascular endothelial cells from patients with AVM and healthy individuals were collected and compared. A dozen genes were found via the selection and analysis of genes associated with both angiogenesis and cell proliferation, which are most relevant processes associated with AVM. Real-time qPCR of such genes showed that the increase in protein levels in the AVM group was statistically significant when compared to those in the control group. A total of seven genes including *ANGPT1*, *ANGPT2*, *DLL4*, *IL6*, *NRG1*, *TGFBR1*, and *VEGFA* were found to be highly expressed. *DLL4*, *TGFB2*, and angiopoietin play an important role in maintaining vascular homeostasis related to angiogenesis and are involved in various processes such as induction of shear stress, hypoxia, and inflammation, which are consistent with AVM [12]. *IL6* affects angiogenesis via the PI3K/Akt pathway [13]. In addition, *NRG1* encoding neuregulin 1 is used as a treatment for myocardial angiogenesis [14]. In general, the genes that were overexpressed and selected were relevant to angiogenesis, which was also consistent with the clinical characteristics of AVM. Besides, the microarray analysis did not demonstrate increased expression of genes related to other pathways and angiogenesis in AVM in this study. A treatment strategy targeting these

seven genes for AVM should be considered. We will continue follow-up studies on drugs or target gene regulators. In conclusion, Seven genes, *ANGPT1*, *ANGPT2*, *DLL4*, *IL6*, *NRG1*, *TGFBR1*, and *VEGFA*, were selected to analyze the pathophysiological mechanism of AVM. These results would provide the foundation for the development of specific drugs or new treatment methods for patients in the future.

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## FINANCIAL DISCLOSURE STATEMENT

None

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## INSTITUTIONAL REVIEW BOARD STATEMENT

This study was approved by the Institutional Review Board of Kyungpook National University Hospital (approval number: KNUH 2020-03-078-001) and was performed in accordance with the Helsinki Declaration.

## INFORMED CONSENT STATEMENT

Informed consent was obtained from all the subjects involved in the study.

## DATA AVAILABILITY STATEMENT

The data presented in this study are available in the manuscript.

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# Growth Outcome and Metabolic Profile of PWS Patients Treated with GH and Differences between AGA and SGA Group

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**Background:** Prader-Willi syndrome (PWS) is a complex genetic disease associated with growth impairment, severe obesity and metabolic dysfunctions. High proportion of PWS patients are born small for gestational age (SGA) than normal children, which also increase the risk of growth impairment and metabolic dysfunction in PWS. We aimed to compare growth outcome and metabolic profiles between SGA and appropriate for gestational age (AGA) PWS patients. **Methods:** Data of 55 PWS children and adults aged more than 2 years old (32 male and 23 female, age 2-18.8 years) from single center were studied. Only patients who were treated with GH were included. The clinical characteristics and laboratory findings were reviewed retrospectively. **Results:** Among 55 subjects, 39 had 15q11-13 deletion and 16 had uniparental disomy (UPD). Twenty (36.3%) were born SGA. All patients received GH treatment, and 11 (20%) discontinued GH treatment. Mean age at GH treatment initiation was 2.5 (range 0.3-12.4) years, and mean duration of treatment was 6.3 (range 1.0-11.3) years. Current height-SDS (-0.36 vs -0.16) and BMI-SDS (1.44 vs 1.33) did not differ between AGA and SGA group. Two patients in SGA group, but none in AGA group had diabetes mellitus. Mean glucose level was also higher in SGA group (100.1 vs 114.4 mg/dL). **Conclusion:** Our report gives an overview of growth profile and metabolic dysfunctions recorded in GH treated PWS patients. Growth profile did not differ between AGA and SGA group. Glucose level was higher in SGA group, so more careful monitoring and prevention for DM will be required in SGA group.

Key words: Prader-Willi syndrome, Growth outcome, SGA

ORIGINAL ARTICLE

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## INTRODUCTION

Prader-Willi syndrome (PWS) is a complex genetic disorder characterized by severe obesity and neurodevelopmental abnormality. PWS results from absence of expression of imprinted genes in the chromosome 15q11-q13 region, caused by deletion of the paternal copy (70%), maternal uniparental disomy (UPD) (25%), an imprinting center defect (1-3%), or balanced translocation [1-3]

It is known that about 90% of patients with PWS have short stature [4]. When growth hormone is not replaced, average adult height in male and female patients with PWS is 155 cm and 148 cm, respectively [5,6]. But when treated with growth hormone (GH) through childhood, children with PWS are able to achieve normal adult height [5,7]. So, recombinant human growth hormone (rhGH) was FDA approved in the United States in 2000 for treatment of short stature and growth failure due to PWS [8].

PWS patients are frequently born small for gestational age (SGA), and body mass index (BMI) of PWS are approximately 15–20% lower than those of their normal mates [9]. SGA is also a risk factor of short adult height. Among SGA children, 8-12% will have a short stature at 2 years of life, and these children have a higher risk of short stature in adulthood [10,11]. So, GH treatment is indicated for children born SGA, who have persistent short stature (less than or equal to –2

Table 1. Clinical characteristics

| Variable | All patients (n=55) | SGA (n=20) | AGA (n=35) | *P* value |
|---|---|---|---|---|
| Male (n, %) | 32 (58.2) | 15 (75.0) | 17 (48.6) | 0.056 |
| Current age (yr) | 9.0±4.0 | 8.8±3.8 | 9.2±4.1 | 0.749 |
| Age at GH initiation (yr) | 2.2±2.6 | 1.6±1.8 | 2.5±2.9 | 0.157 |
| Duration of GH treatment (yr) | 6.3±3.0 | 7.0±2.6 | 5.9±3.0 | 0.176 |
| Gestational age (wk) | 38.6±1.9 | 38.8±1.3 | 38.5±2.2 | 0.445 |
| Birth weight (kg) | 2.68±0.46 | 2.38±0.29 | 2.85±0.45 | <0.001 |
| Genetic causes | | | | 0.466 |
| deletion (n, %) | 39 (70.9) | 13 (65.0) | 26 (74.3) | |
| uniparental disomy (n, %) | 16 (29.1) | 7 (35.0) | 9 (25.7) | |

SGA, small for gestational age; AGA, adequate for gestational age; GH, growth hormone.

SDS according to sex and age for general population for sex and population) at the age of 3–4 years [12].

In this study, we compared growth and metabolic profile and effect of GH between SGA and AGA PWS.

## METHODS

### Subjects and method

We obtained data of PWS children who visited our hospital from 2007-2020. Inclusion criteria were genetically confirmed PWS patients aged more than 2 years who used GH more than 6 months.

As our routine clinical practice for PWS patients, height (Ht), weight, body mass index (BMI), glucose, HbA1c, and lipid level were checked at regular follow-up with 3-6 months interval. By retrospective chart review, we obtained anthropometric parameters and laboratory findings at the time of GH initiation and at last follow up. All standard deviation scores (SDS) of anthropometric measurements were calculated using a Korean growth standard [13]. SGA was defined by birthweight below the 10th percentile for babies of the same gestational age and sex. Adequate for gestational age (AGA) was defined by birthweight 10-90th percentile for babies of the same gestational age and sex. Obesity was defined by BMI more than 95th percentile for the children of the same age and sex. Diabetes mellitus (DM) was defined as fasting blood glucose ≥126 mg/dL or random glucose ≥200 mg/dL or taking DM medication. Dyslipidemia was defined as total cholesterol ≥200 mg/dL or triglyceride ≥150 mg/dL or LDL cholesterol ≥130 mg/dL, or taking lipid lowering medication.

### Statistical analysis

All data are presented as n(%) for the mean±standard deviation. Differences in anthropometric measurements and treatment response between groups were compared using Student t test and Wilcoxon rank sum test. All statistical analyses were performed using SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA), and *P* values <0.05 were considered statistically significant.

## RESULTS

### Clinical characteristics

Total fifty-five patients were enrolled in this study. Twenty were born SGA and thirty-five were born AGA. Thirty-two (58.2%) were male, and age was. 9.0±4.0 years. Patients used growth hormone for 6.3±3.0 years. Genetic causes were deletion in thirty-nine (70.9%) and uniparental disomy in 16 (29.1%) patients. There were no differences in clinical characteristics between SGA and AGA group (Table 1).

### Anthropometric characteristics and current laboratory data

In all subjects, Ht-SDS at GH initiation and current Ht-SDS were -0.91±1.60 and -0.29±1.34, respectively. BMI-SDS at GH initiation and current BMI-SDS were 1.64±1.97 and 1.33±2.42, respectively. HbA1c and total cholesterol were 5.4±0.3% and 184.0±29.3 mg/dL, respectively. SGA children had higher glucose level than AGA children (114.4±27.8 vs. 100.1±16.0 mg/dL. *P*=0.019). There were no differences in other anthropometric and laboratory parameters between SGA and AGA group (Table 2).

### GH treatment effect and metabolic dysfunction

We compared growth effect and metabolic profile. Current Ht-SDS and ΔHt-SDS were -029±1.34 and 0.66±1.50, respectively. ΔBMI-SDS was -0.40±1.88. There were no differences between SGA with AGA group (Table 3).

**Table 2.** Anthropometric characteristics and current laboratory data

| Variable | All (n=55) | SGA (n=20) | AGA (n=35) | *P* value |
|---|---|---|---|---|
| Ht-SDS at GH initiation | -0.91±1.60 | -1.23±1.26 | -0.77±1.73 | 0.394 |
| Current Ht-SDS | -0.29±1.34 | -0.16±1.16 | -0.36±1.44 | 0.613 |
| BMI-SDS at GH initiation | 1.64±1.97 | 1.33±2.42 | 1.78±1.85 | 0.693 |
| Current BMI-SDS | 1.40±1.19 | 1.33±1.20 | 1.44±1.21 | 0.752 |
| HbA1c (%) | 5.4±0.3 | 5.46±0.43 | 5.42±0.24 | 0.669 |
| Glucose (mg/dL) | 105.4±22.0 | 114.4±27.8 | 100.1±16.0 | 0.019 |
| Triglyceride (mg/dL) | 111.2±66.2 | 103.8±39.8 | 115.6±72.4 | 0.507 |
| Total Cholesterol (mg/dL) | 184.0±29.3 | 184.7±28.8 | 182.7±30.7 | 0.803 |
| LDL (mg/dL) | 105.9±25.2 | 104.6±24.3 | 106.6±26.0 | 0.783 |

Ht, height; SDS, standard deviation score; BMI, body mass index; LDL, low density lipoprotein.

**Table 3.** GH treatment effect and metabolic dysfunction

| Variable | All patients (n=55) | SGA (n=20) | AGA (n=35) | *P* value |
|---|---|---|---|---|
| Current Ht-SDS | -029±1.34 | -0.16±1.16 | -0.36±1.44 | 0.613 |
| ΔHt-SDS (current–GH initiation) | 0.66±1.50 | 1.05±1.32 | 0.50±1.56 | 0.273 |
| ΔBMI-SDS (current–GH initiation) | -0.40±1.88 | -0.55±3.03 | -0.33±1.27 | 0.884 |
| DM (n, %) | 2 (3.6) | 2 (10.0) | 0 | 0.128 |
| Overweight/obesity (n, %) | 34 (61.8) | 13 (68.4) | 21 (65.6) | 0.838 |
| Dyslipidemia (n, %) | 21 (38.2) | 7 (35.0) | 14 (40.0) | 0.714 |

Ht, height; SDS, standard deviation score; BMI, body mass index; DM, diabetes mellitus.

## DISCUSSION

Our study compared GH treatment effect and metabolic profile between SGA and AGA PWS children. Our study is, as we know, the first study which compared GH treatment effect between SGA and AGA PWS children. Our study involved relatively large number of patients in single center, which can maximize homogeneity in patients or treatment.

Growth effect of GH in SGA children is thought to be lesser than in AGA children. Miazza et al. [14] compared growth effect of GH in SGA and AGA children. They reported that height velocity was higher in AGA-GHD children during the first year of treatment. Nonetheless, GH treatment in short children born SGA has shown a beneficial, growth-promoting effect in both the short-and long-term [15].

Our study has several limitations. We did not compare GH-treated with not-GH treated group, because most of PWS children in our clinic received GH therapy. And we did not compare predicted adult height or final height. These should be further explored to get effect of GH more precisely. For metabolic effect, we did not compare metabolic profile before and after GH, and compared only current metabolic profile. But in our data, SGA children had higher fasting glucose level, and only SGA patients had DM. This gives strength to our hypothesis that SGA PWS children are prone to metabolic disorder, especially impaired glucose metabolism

In conclusion, our study describes growth response to GH and metabolic profiles, and differences among SGA and AGA PWS groups. The results of this study provide useful suggestions to make better decisions to start and optimize GH treatment in PWS children.

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## FINANCIAL DISCLOSURE STATEMENT

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## CONFLICTS OF INTEREST

None

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# Information Authors

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## STUDY CONDUCT AND POLICIES

### 1. Research and publication ethics

In regard to the process of reviewing and assessing the manuscripts, including the ethical guidelines and plagiarism/ duplicate publication and study misconduct, it should conform to the ethical guidelines specified in the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (http://www.icmje.org/recommendations/), which were established by the International Committee of Medical Journal Editors (ICMJE). For the policies on the research and publication ethics not stated in this instruction, Good Publication Practice Guidelines for Medical Journals 3rd (Korean Association of Medical Journal Editors, KAMJE; http://kamje.or.kr/intro.php?body=publishing_eth-ics) or Guidelines on Good Publication (Committee on Publication Ethics, COPE; http://publicationethics.org/resources/guidelines) can be applied.

### 2. Disclosure of conflicts of interest

Financial sponsorship should be stated in the conflicts of interest. Any other financial support associated with the study, including stocks or consultation arrangements with pharmaceutical companies should be stated at the end of the text, under a subheading "Conflicts of interest."

### 3. Statement of informed consent

Human study must conform to ethical standards, and be approved by the appropriate Institutional Review Board (IRB). A statement concerning IRB approval and consent procedures must appear at the beginning of the "Methods" section. Any systematic data gathering effort in patients or volunteers must be approved by an IRB or adhere to appropriate local/national regulations. Authors may be questioned about the details of consent forms or the consent process. On occasion, the Editor-in-Chief may request a copy of the approved IRB application from the author.

### 4. Statement of human and animal right

Clinical research studies must state that the work was done in accordance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the Declaration of Helsinki in 1975 (revised in 2013; http:// www.wma.net/en/30publications/10policies/b3/index. html). Clinical studies that do not meet the Declaration of Helsinki will not be considered for publication. Human subjects must not be identifiable. Patients' name, initial, hospital number, date of birth, or other protected health- care information must not be disclosed.

Animal research studies must state that the work was performed according to National or Institutional Guide for the Care and Use of Laboratory Animals, and the ethical treatment of all experimental animals must be observed. For experimental studies involving client-owned animals, authors must also include a statement on informed consent from the client or owner.

### 5. Authorship

The JIG follows the recommendations for authorship by the ICMJE, 2019 (http://www.icmje.org/icmje-recommendations.pdf) and Good Publication Practice Guidelines for Medical Journals 3rd Edition (KAMJE, 2019, https://www.kamje.or.kr/board/view?b_name=bo_publication&bo_id=13&per_page=). Authorship credit must be based on 1) substantial contributions to the concept and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; 3) final approval of the version to be published; and 4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors must meet the above four conditions.

A corresponding author should be designated when there are two or more authors. The corresponding author is primarily responsible for all issues to the editor and audience. Any comment of the corresponding author is regarded as opinion of all coauthors.

When a large, multicenter group has conducted the work, the group should identify the individuals who accept direct responsibility for the manuscript. When submitting a manuscript authored by a group, the corresponding author should clearly indicate the preferred citation and identify all individual authors as well as the group name. Journals generally list other members of the group in the Acknowledgments. Acquisition of funding, collection of data, or general supervision of the research group alone does not constitute authorship.

### 6. Originality and duplicate publication

All submitted manuscripts should be original and should not be considered by other scientific journals for publication at the same time. No part of the accepted paper should be duplicated in another scientific journal without permission by Editorial Board. If duplicate publications related to the papers of this journal are detected, sanctions against authors range from requesting their institutions to assess the facts, requesting a Letter to the Editor-in-Chief acknowledging the error and voluntarily withdrawing a paper, to a ban on publication in JIG up to 3 years.

### 7. Sex and gender equity in research

We encourage the use of the Sex and Gender Equity in Research (SAGER) guidelines (http://doi.org/10.1186/s41073016-0007-6) for reporting of sex (biological factor) and gender (identify, psychosocial or cultural factors) information in study design, data analysis, and manuscripts writing.

## MANUSCRIPT PREPARATION AND FORMAT

*JIG* follows the Open Access Journal policy and publishes the following article types:

- Original articles
- Review article
- Editorial
- Brief report
- Image
- Letters to the editor

The other form could be submitted under the approval of the editorial board.

### 1. General principles

1) Manuscripts should be prepared using MicroSoft Word (.doc). All portions of the manuscript should be double-spaced with the font size of 12 with 3-cm blank margin in both sides, top and bottom of A4-sized paper (21 × 30 cm). The numbers on each page of the manuscript should be placed at the center of the bottom in a sequential manner starting from the title page.
2) Manuscripts should be submitted in English. Medical terminology should be written based on the most recent edition of English-Korean Korean-English Medical Terminology, published by the Korean Medical Association.
3) Acronyms should only be used when absolutely necessary for clarity. In cases in which the use of acronyms is desirable due to the repetition, the acronym should be expressed in a parenthesis when the corresponding terminology first appears in the manuscript.
4) Laboratory measurements should be used in International System of Units (SI) units. However, in some cases, non-SI units (conventional units) can also be used in a versatile manner. But the usage of units should be consistent.
5) The manuscript should be arranged in the following order: title page, abstract and keywords, main text (introduction, methods, results anddiscussion), conflicts of interest, acknowledgments, references, tables and figures.
6) All authors are encouraged to provide Open researcher and contributor ID (ORCID). Additional information about ORCID is available at http://orcid.org/.

### 2. Original articles

*Title page and abstract*

1) The title page should be as follows: the title of the manuscript, a short running title less than 50 characters, names of all authors and their current affiliations. In cases in which the authors belong to multiple affiliations, the affiliations during the study being reported should be matched to the authors' names using a superscript of Arabic numerals
2) The title of the manuscript should be no longer than 20 English words. The first letter of each word of the title must be capitalized. Acronyms should not be used in title except for special situations.
3) At the bottom of the title page, the corresponding author's name, address, and e-mail address should be indicated.
4) Provide the word count for the text only, excluding abstract, acknowledgments, figure legends, and references.
5) The number of figures and tables should be provided in the title page.
6) The word count for the abstract should be no more than 250 words, consisting of four sections: Background, Meth-

ods, Results, and Conclusion.

7) Three to 10 keywords relevant to the content of a manuscript should be attached after the abstract. In principle, the keywords should be found in MeSH terms of the Index Medicus (http://www.nlm.nih.gov/mesh/MBrowser.html).
8) Author names and affiliations: On the title page, write Each author's name (first name, middle name, and family name) followed by the highest academic degree, full institutional mailing addresses and ORCID (Open Researcher and Contributor ID) number. Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled "current address." At a minimum, the address must include the author's current institution, city, and country. If an author has multiple affiliations, enter all affiliations on the title page.

*Main text*

1) ***The main text should be structured as Introduction, Methods, Results, and Discussion.*** Use headings and subheadings in the Methods section and Results section. Every reference, figure, and table must be cited numerically in the order mentioned in the text.
2) ***Introduction:*** Present the research purpose briefly and clearly, together with only the background information that is relevant to the purpose
3) ***Methods:*** Materials, methods, and study design should be presented in detail. In experimental research, methods should be described in such a manner that the experiments can be reproduced by the readers. A statement concerning IRB approval and consent procedures must appear at the beginning of the Methods section. The description for the reagents, kits machines used in the experiment should be precise with full descriptions for the kit number, company name, city and the country of its origin.
4) ***Results:*** A detailed description of the study results should be clearly arranged in a logical manner. In cases in which tables are used, the contents described in tables should not be redundantly described in the main text, but the important trends and points should be emphasized. Insertion of references with previously published data is not allowed in results section. Description of previously reported data or personal opinion should be mentioned in discussion section. In tables, figures, virgule constructions and within parentheses, 'minute' should be described with 'min' and 'hour with 'hr', and no 's' should be added to them
5) ***Discussion:*** New and important observations should be emphasized. A redundant description of the results is not acceptable. The significance and limitation of the observed findings should be described. There should be a link between the conclusions and the goals of the study. Conclusions not adequately supported by the data must be avoid.

*Conflicts of interest*

Any potential conflict of interest relevant to the manuscript is to be described. If there are no conflicts of interest, authors should state that none exist.

*Acknowledgments*

The acknowledgments should be presented after the main text and before the reference list. Acknowledgments should contain brief statements of assistance, financial support, and prior publication of the study in abstract form, where applicable. Any other matters associated with research funds, facilities and drugs which were used in the current manuscript should also be given in the Acknowledgments

*References*

1) References should be listed in the sequence cited in the paper, and sequential numbers should be attached in the middle or at the end of the corresponding sentences in the body of the text. The reference list should be given at the end of the document, after the main text and acknowledgments (if applicable) and before the tables.

   Original articles are limited to 40 references. Reference numbers in the text should appear in chronological order in normal type and in square brackets, e.g., "In the study by Norton et al. [23]…".
2) The names of all authors must be listed by the last name and the initials of first and middle names in each reference. List all authors when the number of authors is 6 or less. If 7 or more, list the first 6 authors and add "et al." Inclusive page numbers must be provided. The notation of academic journal names should be used with acro-

nyms approved by Index Medicus. (Available form:http://www.nlm.nih.gov/archive/20130415/tsd/serials/lji.html)

3) All the references should be described in the following format:

***Journals:*** authors' names (list the first 6 authors and add "et al."), title, journal name, year, volume, and page number

e.g., Amos-Landgraf JM, Ji Y, Gottlieb W, Depinet T, Wandstrat AE, Cassidy SB, et al. Chromosome breakage in the Prader-Willi and Angelman syndromes involves recombination between large, transcribed repeats at proximal and distal breakpoints. Am J Hum Genet 1999;65:370-86.

***Book:*** authors' name(s), title, number of editions, place of publication, publisher, year of publication, and page numbers.

e.g., Imura H. The pituitary gland. 2nd ed. New York: Raven Press; 1974. p. 453-90.

***Chapter in a book:*** authors' name(s), title, number of editions, place of publication, publisher, year of publication, chapter number and title, and page numbers.

e.g., Speroff L, Fritz MA. Clinical gynecologic endocrinology and infertility. 7th ed. Philadelphia: Lippincott Williams & Wilkins; 2005. Chapter 29, Endometriosis; p. 1103-33.

***Dissertation:*** author, title [book type], place of publication, publisher, year of publication.

e.g., Kaplan SJ. Post-hospital home health care: the elderly's access and utilization [dissertation]. [St. Louis]: Washington University; 1995.

***Web sites:*** author(s), title [type of medium], place of publication, publisher, year of publication [date of update, date of citation], URL.

e.g., International Union of Biochemistry and Molecular Biology. Recommendations on biochemical & organic nomenclature, symbols & terminology etc. [Internet]. London: University of London, Queen Mary, Department of Chemistry; 2006 [updated 2006 Jul 24; cited 2007 Feb 22]. Available from: http://www.chem.qmul.ac.uk/iubmb/.

***Conference proceedings:*** author(s), paper title, In: editor(s), conference title, the year, place, place of publication, pub- lisher, year of publication, and page numbers.

*Tables*

1) Tables should be double-spaced and inserted on a separate page at the end of the text document, with the table number, table title and legend given above the table.
2) Titles of tables should be concise using a phrase and a clause. The first letter of each word of the title should be capitalized.
3) The numbers should be allocated accordingly in order in which the table was quoted in the main text.
4) For acronyms, provide the full names below the corresponding table. Symbols should be marked with small alphabet letters in the order of its usage, such as, a, b, c, d, e with their respective descriptions in the footnote.
5) Tables should be easy to understand while functioning independently.
6) Unnecessary longitudinal lines should not be drawn. Horizontal lines should be refrained from being used as much as possible

*Figures and figure legends*

1) Submit the figures separately from the main text. The resolution power of the pictures and photographs is expected to exceed 300 dpi. Figures should be included with online submissions, either as JPEG, GIF, TIFF, BMP, PICT with RTF manuscripts or embedded in the PDF file.
2) If two or more figures require the same number, Arabic numerals should be followed by letters (e.g., Fig. 1A, Fig. 1B).
3) An author may request pictures to be printed in color, but the cost of this will be charged to the author.
4) Assign sequential numbers (Arabic numerals) in the order referenced in the paper.
5) Figures legends should be written at the end of the manuscript, should be described with complete sentences rather than incomplete phrases or a clause. The expansions for the abbreviations used within the figure should be placed in the legend.
6) For microphotographs, describe the dyeing method and magnification ratio.
7) The description of footnotes below the figure should follow the order of that of acronyms and then symbols. Symbols should be marked with small alphabet letters in the order of its usage, such as, a, b, c, d, e, in superscript.

*Supplemental data*

Nonessential tables and figures may accompany articles as

online-only supplemental files. All online-only supplementary files should be combined in one document file (whenever possible) and uploaded separately during the submission process. This file must be clearly labeled as "Online-Only Supplemental Material." In addition, supplemental onlineonly files must be referenced in the main text of the manuscript at least once (e.g., "Supplemental Table S1").

All online-only supplemental files are subject to review, but such files will not be copyedited or proofread by EnM production staff. As such, authors are encouraged to review their supplemental files carefully before submitting them.

Lists that include names of principal investigators or writing groups may also be submitted as online-only supplements if they exceed 150 words. Otherwise, the names of principal investigators or writing groups should be listed in an appendix at the end of the main document, before the references.

### 3. Review article

A review article is a review focusing on a specific title and commissioned by the Publication Committee for publishing. Manuscripts submitted as review articles will be subjected to the same review process as original research articles. Instructions for original articles should be followed for review articles. A review article should include an abstract not exceeding 200 words, for which the number of references should not exceed 60.

### 4. Editorial

Editorials are commissioned for the purpose of commenting on a specific paper published by the journal, not to reflect the views of the Society. There is no limitation on the format. But an editorial should be written in no more than four pages (A4) with the number of references limited to 20.

### 5. Brief report

Short communications of original research are published as case report. The purpose of the category is to permit publication of very important, high-quality mechanistic studies that can be concisely presented. These manuscripts should include a short nonstructured abstract (150 words maximum), Introduction, Methods, Results, and Discussion.

The total manuscript length should not exceed 1,200 words, excluding references and abstract. Brief reports can include a maximum of 20 references and two figures or tables.

### 6. Image

Images that may help make clinical decisions while being interesting and educational in terms of the treatment of endocrinology and metabolism should be prepared with a manuscript. The manuscript should not be more than one page (A4), with the number of references limited to five.

### 7. Letters to the editor

A letter should contain constructive criticisms or comments on a specific paper published by the journal within the previous 6 months. The manuscript should be no more than one page (A4), with the number of references limited to five

## MANUSCRIPT SUBMISSION, REVIEW AND PUBLICATION

1) Authors should submit manuscripts via the electronic manuscript management system for JIG (https://acoms.kisti.re.kr/journal/intro.do?page=logo&journalSeq=J000161). Please log in as a member of the system and follow the directions. The revised manuscript should be submitted through the same web system.
2) Submitted manuscripts are first reviewed by journal editors. If a manuscript fails to comply with the submission guidelines or the checklist (JIG Submission Checklist), it will be rejected for review and then returned to the author, to be re-written and re-submitted according to the submission guidelines.
3) All submitted manuscripts are peer-reviewed by three anonymous reviewers who are specialists in the relevant field, and the review period would not exceed 3 months. Publishing will be determined based on the review result and revisions or additions will be recommended to the authors as appropriate. Editorial Board determines whether manuscripts are acceptable. If changes are needed, the authors are recommended to revise and amend the manuscripts within 3 months. If the revised manuscript is not returned within this period, it will be deemed that the author has decided not to pursue publication.
4) If necessary, the Editorial Board may consult statisticians during the review process.
5) Authors of a revised manuscript must describe on a line by line basis how the manuscript was revised according to the instructions of the referees.
6) The finally accepted manuscript will be reviewed by manuscript editor for the consistency of the format and the completeness of references. The manuscript may be revised according to the style guides of the journal.

7) Before publication, the galley proof will be sent via email to the corresponding author for approval. Galley changes must be returned within 48 hours. Changes should be limited to those that affect the accuracy of the information presented
8) The ORCID ID will be displayed in the published article for any author on a manuscript who has a validated ORCID ID when the manuscript is accepted.

## OTHERS

1) If it is necessary to revise a manuscript, the Publication Committee may do so insofar as it does not impact the original text, and according to its editing policy on wording and formats.
2) The submission fee (₩10,000) will be assessed for articles submitted beginning June 2020. The fee will not be refunded regardless of editorial decision (including editorial rejection). Fees should be paid by credit card. If a credit card cannot be used, a check or wire transfer can be accepted. There is no author's publication-related fee since all article processing cost is supported by Korean Interdisciplinary Society of Genetic & Genomic Disorders until there is a policy change. However, if designing, plating or special printing is required for the publication, the actual expenses for these shall be paid for by the author.
3) Publication by the journal shall be deemed to mean that the author has consented that the copyright thereof will be transferred to the journal.

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