

Gene polymorphisms in leptin and its receptor and the response to growth hormone treatment in patients with idiopathic growth hormone deficiency

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Abstract. This study aimed to investigate the relationships between genetic polymorphisms of leptin/receptor genes and clinical/biochemical characteristics in children with growth hormone deficiency (GHD). Ninety-three GHD children and 69 age-matched normal controls were enrolled. Anthropometric measurements, bone age, and laboratory test results were obtained. Polymorphisms in the *LEP* gene promoter locus (LEP-2548, rs7799039) and *LEPR* genes (K109R, rs1137100 and Q223R, rs1137101) were analyzed using PCR-RFLP. The serum leptin levels were measured using an ELISA kit. The median height and BMI z-scores of all GHD subjects were -2.20 and -0.26 , respectively, and those of normal controls were -0.30 and -0.13 , respectively. The serum leptin levels were similar between GHD subjects and normal controls ($p = 0.537$), but those were different between the complete GHD (6.97 ng/mL) and partial GHD (4.22 ng/mL) groups ($p = 0.047$). There were no differences in the genotypic distributions of LEP-2548, LEPR K109R, and Q223R between GHD subjects and normal controls. However, GHD subjects with the G allele at LEP-2548 showed higher IGF-1 ($p = 0.047$) and IGFBP-3 SDSs ($p = 0.027$) than GHD subjects with the A allele. GHD subjects with the G allele at LEPR Q223R showed lower stimulated GH levels ($p = 0.023$) and greater height gain after 1 year of GH treatment ($p = 0.034$) than GHD subjects with the A allele. In conclusion, leptin/leptin receptor genes are suggested to have the role of growth-related factors, which can affect various growth responses in children who share the same disease entity.

Key words: Leptin, Leptin receptor, Polymorphism, Growth hormone deficiency

SHORT STATURE (SS) is a common and heterogeneous condition which implies various diseases. As is already known, the growth hormone (GH) – insulin-like growth factor 1 (IGF-1) axis is majorly important in statural growth. However, the prevalence of growth hormone deficiency (GHD) is estimated to be as low as 1:10,000 [1]. Among total patients with short stature, disorders of the GH–IGF-1 axis accounts for only 2%,

while estimated frequency of idiopathic short stature (ISS), which implies a diagnosis of exclusion, is over 60% [2]. Therefore, there have been curiosities about related factors to growth beyond the GH – IGF-1 axis.

Recently, from genome-wide association study (GWAS) to next-generation sequencing (NGS), the rapid progress of molecular technologies has helped us identify novel genetic causes of SS or single nucleotide polymorphisms (SNPs) of various genes [3]. Yang *et al.* reported that the contribution of common SNPs to the height phenotype is 40% based on GWAS [4]. In a Korean population, 15 genetic loci influence adult height and five genetic loci are associated with idiopathic short stature (ISS) [5]. To date, various candidate genes and SNPs that could explain the height phenotype have been

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proposed [2, 6, 7]. Based on several studies, the leptin and its receptor were also considered among these candidates [8-11]. The major role of leptin in growth is known to be in the stimulation of proliferation and differentiation of chondrocytes [12].

In children with GHD, growth velocity in the first year of the GH treatment was significantly lower in the underweight cohort than in the normal weight or obese cohorts [13]. Healthy children with relatively large fat mass require less GH to maintain normal growth than children with lower fat mass [14]. Therefore, fatness and growth may be closely connected by some messengers, and leptin has been proposed as one of these messengers. Serum leptin, which is well correlated with the body mass index (BMI), has roles in the satiety response, onset of puberty, and differentiation of chondrocytes in the growth plate [15]. Increased circulating leptin levels in obesity contribute to robust linear growth, which is considered as GH-independent growth [16].

However, the results of correlation studies on the relationships between leptin and clinical/biochemical parameters, such as fat mass, lean mass [17-19], IGF-1 levels [20], and GH secretions [14, 21], are different, thereby implying the possible role of polymorphisms in leptin or its receptor. In GHD patients, the adverse metabolic effects of increased fat mass may be associated with elevated leptin levels [14, 21]. However, variable levels of circulating leptin in patients with GHD have also been observed [17, 18]. Therefore, we investigated the role of polymorphisms in leptin or leptin receptor genes in GHD patients. The aim of this study was to confirm leptin (*LEP*-2548) and leptin receptor (*LEPR* K109R and Q223R) gene polymorphisms and to investigate the contribution of gene polymorphisms to clinical/biochemical characteristics in children with GHD.

Subjects and Methods

Subjects

Ninety-three GHD subjects (58 males and 35 females) aged 8.7 (4.0 ~ 14.5) years old and 69 age-matched normal controls (20 males and 49 females) aged 8.1 (4.7 ~ 11.1) years old who visited the Hallym Medical Center for their growth check-up were enrolled. GHD was diagnosed when the subject satisfied all of the following criteria: height <3rd percentile (z score of -1.88); peak GH level after stimulation <10 ng/mL; delayed bone age; full term (gestational age between 37 and 41 weeks); normal birth weight (between 2.5 and 4.2 kg); and no documented systemic, endocrine, nutritional, or chromosomal abnormalities. GHD was classified according to the peak GH level after stimulation as follows: complete GHD, peak GH < 5 ng/mL; and partial GHD, 5 ng/mL ≤ peak

GH < 10 ng/mL. Two different stimuli were used in GH stimulation as follows: clonidine, L-dopa, arginine, insulin, or glucagon. GHD subjects with organic brain lesion shown on MRI were excluded from this study. The inclusion criteria for the normal controls were as follows: 3rd percentile ≤ height ≤ 97th percentile; 5th percentile ≤ BMI ≤ 84th percentile; the difference between the bone age and chronological age should be less than two years. Medical records were reviewed to check baseline anthropometric measurements (height and weight), bone age, and laboratory test results, including GH stimulation test, IGF-1, and IGF-binding protein 3 (IGFBP-3) levels. In GHD subjects, anthropometric data after 1 year of GH treatment were also reviewed.

Height was measured twice to the first decimal place with a Harpenden stadiometer (Holtain Ltd., Crosswell, UK) and weight was measured to the first decimal place with a digital scale (150A; Cas Co. Ltd., Seoul, Korea). The z-scores for height, weight, and BMI were calculated using the 2017 Korean growth standard [22]. Rohrer index was also calculated [23]. Bone age was determined by a pediatric endocrinologist using the Greulich and Pyle hand standards [24]. Pubertal status was examined by a pediatric endocrinologist using Tanner stage. Written informed consent was obtained from all subjects and their parents before study enrollment. The institutional review boards (IRB) of Hallym Medical Center (IRB# KANGDONG 2017-07-002-001) approved this study.

Gene sequencing for *LEP*-2548, *LEPR* K109R, and Q223R

Blood samples from subjects (both GHD and normal controls) who met the inclusion criteria and agreed to participate in this study were collected in EDTA-containing tubes. Genomic DNA was isolated from peripheral blood leukocytes according to the manufacturer's instructions (Qiagen, QIAGEN GmbH, Hilden, Germany).

Polymorphisms in the *LEP* gene promoter locus (*LEP*-2548, rs7799039) and *LEPR* genes (K109R, rs1137100 and Q223R, rs1137101) were selected in this study based on a previous report which SNPs demonstrating relationships between SNPs and growth [11]. SNP genotyping was performed using PCR-RFLP. The digested products were separated on a 2.5% agarose gel solution and stained with ethidium bromide. The genotypes were confirmed by DNA sequencing analysis. The primer sequences and PCR protocols are listed in Supplemental Table 1.

Serum leptin analysis

Serum was obtained from the blood samples of the GHD subjects and stored at -80°C before being assayed.

Table 1 Clinical and biochemical characteristics of subjects

	GHD (<i>n</i> = 93)	Complete GHD (<i>n</i> = 27)	Partial GHD (<i>n</i> = 66)	Normal Control (<i>n</i> = 69)
Male:Female (<i>n</i>) ¹⁾	58:35	16:11	42:24	20:49
Age (year)	8.7 (4.0 ~ 14.5)	9.4 (4.7 ~ 14.5)	8.6 (4.0 ~ 13.4)	8.1 (4.7 ~ 11.1)
Baseline Ht z-score ¹⁾	-2.20 (-3.55 ~ -1.88)	-2.17 (-3.0 ~ -1.88)	-2.22 (-3.55 ~ -1.89)	-0.29 (-1.66 ~ 1.47)
Baseline BMI z-score ²⁾	-0.36 (-3.07 ~ 1.50)	0.36 (-2.34 ~ 1.50)	-0.45 (-3.07 ~ 1.34)	-0.16 (-1.62 ~ 1.03)
Baseline Rohrer index ^{1,2)}	0.014 (0.010 ~ 0.019)	0.015 (0.011 ~ 0.018)	0.014 (0.010 ~ 0.019)	0.013 (0.011 ~ 0.016)
Baseline BA-CA (yrs) ¹⁾	-2.1 (-5.5 ~ 0.0)	-2.1 (-5.5 ~ 0.0)	-2.1 (-4.3 ~ 0.0)	-0.7 (-2.0 ~ 1.9)
Ht z-score after 1 yr of GH treatment	-1.55 (-2.56 ~ -0.62)	-1.55 (-2.18 ~ -0.62)	-1.55 (-2.56 ~ -1.01)	—
BMI z-score after 1 yr of GH treatment	-0.50 (-3.33 ~ 1.40)	-0.06 (-1.67 ~ 1.33)	-0.53 (-3.33 ~ 1.40)	—
BA-CA after 1 yr of GH treatment	-1.4 (-4.6 ~ 0.9)	-1.25 (-3.3 ~ 0.9)	-1.40 (-4.6 ~ 0.7)	—
Baseline IGF-1 SDS ¹⁾	-0.98 (-2.15 ~ 0.72)	-0.98 (-2.15 ~ 0.72)	-1.11 (-2.15 ~ -0.02)	-0.65 (-1.74 ~ 1.14)
Baseline IGFBP-3 SDS ¹⁾	1.94 (-0.88 ~ 6.50)	1.94 (-0.88 ~ 6.50)	1.64 (-0.23 ~ 4.65)	2.56 (0.40 ~ 6.14)
IGF-1 SDS after 1 yr of GH treatment	0.03 (-2.40 ~ 7.01)	-0.13 (-1.27 ~ 7.01)	0.28 (-2.40 ~ 4.52)	—
IGFBP-3 SDS after 1 yr of GH treatment	3.51 (-1.46 ~ 6.99)	3.51 (0.29 ~ 6.82)	3.51 (-1.46 ~ 6.99)	—
Baseline serum level of leptin (ng/mL) ²⁾	4.35 (0.53 ~ 31.04)	6.97 (0.74 ~ 31.04)	4.22 (0.53 ~ 24.15)	3.83 (1.29 ~ 21.71)

Data were expressed as median and ranges (minimum, maximum).

¹⁾ *p*-value <0.05, GHD vs. normal control

²⁾ *p*-value <0.05, complete GHD vs. partial GHD

The serum leptin levels were measured using an ELISA kit (Cat. # EZHL-80SK; Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation were 2.2% and 3.4%, respectively.

Statistical analysis

The data were expressed as medians and ranges (minimum and maximum). The Mann-Whitney U-test was used to compare the clinical and biochemical data of the two groups (GHD vs. normal control or complete GHD vs. partial GHD). The allele frequencies between the two groups were compared using a chi-squared test. Spearman's correlation analysis between clinical and biochemical data was performed. The Hardy-Weinberg equilibrium was tested using the chi-squared method. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). A *p*-value <0.05 was considered statistically significant.

Results

Clinical and biochemical characteristics of subjects (Table 1)

The median height z-score of all GHD subjects was -2.20, and it did not differ between the complete and partial GHD groups (*p* = 0.436). The degree of bone age

delay, IGF-1 SDS, and IGFBP-3 SDS were similar between the complete and partial GHD groups. However, the median BMI z-score of complete GHD subjects (0.36) was higher than that of partial GHD subjects (-0.45, *p* = 0.005). After 1 year of GH treatment, the Δ height z-score (height z-score at 1yr of GH treatment - height z-score at baseline) did not differ significantly between the complete (median value: 0.76) and partial (0.66) GHD groups, while the Δ BMI z-score (BMI z-score at 1yr of GH treatment - BMI z-score at baseline) was higher in the complete GHD (-0.34) than in the partial GHD subjects (-0.07, *p* = 0.002). However, there were no differences in the Δ IGF-1 SDS and IGFBP-3 SDS between the complete and partial GHD groups.

The serum leptin levels were similar between the GHD (4.35 ng/mL) and normal control subjects (3.83 ng/mL, *p* = 0.537), but there was a significant difference between the complete GHD (6.97 ng/mL) and partial GHD (4.22 ng/mL) groups (*p* = 0.047). The serum leptin levels and BMI z-score were positively correlated in all subjects (*r* = 0.445, *p* < 0.001) and this relationship was constant regardless of the GHD status.

The distribution of SNPs in LEP-2548, LEPR K109R, and LEPR Q223R (Tables 2 and 3)

The dominant allele of each SNP was A, G, and G at LEP-2548, LEPR K109R, and Q223R, respectively, as in the reference database. There were no differences in the

Table 2 The distributions of SNPs of LEP-2548, LEPR K109R, and LEPR Q223R

		GHD (<i>n</i> = 93)	Complete GHD (<i>n</i> = 27)	Partial GHD (<i>n</i> = 66)	Normal Control (<i>n</i> = 69)
LEP-2548	AA/AG/GG (%)	63.4/32.3/4.3	74.1/22.2/3.7	59.1/36.4/4.5	69.6/30.4/0.0
	AA/AG + GG (%)	63.4/36.6	74.1/25.9	59.1/40.9	69.6/30.4
K109R	GG/GA/AA (%)	62.4/35.5/2.2	66.7/33.3/0.0	60.6/36.4/3.0	66.7/27.5/5.8
	GG/GA + AA (%)	62.4/37.6	66.7/33.3	60.6/39.4	66.7/33.3
Q223R	GG/GA/AA* (%)	72.0/25.8/2.2	92.6/7.4/0.0	63.6/33.3/3.0	76.8/21.7/1.4
	GG/GA + AA* (%)	72.0/28.0	92.6/7.4	63.6/36.4	76.8/23.2

* *p*-value <0.05, complete GHD vs. partial GHD

Table 3 Comparison of clinical characteristics between groups according to genotype in GHD subjects

LEP-2548	AA (<i>n</i> = 59)	AG/GG (<i>n</i> = 34)	<i>p</i> -value
IGF-1 SDS	-1.19 (-2.15 ~ 0.28)	-0.79 (-1.79 ~ 0.72)	0.047
IGFBP-3 SDS	1.73 (-0.88 ~ 5.98)	2.45 (0.27 ~ 6.50)	0.027
LEPR223	GG (<i>n</i> = 67)	GA/AA (<i>n</i> = 26)	<i>p</i> -value
Stimulated GH (ng/mL)	5.7 (0.6~9.9)	6.3 (4.3~9.9)	0.023
Height gain after GH treatment	0.74 (0.07~ 1.40)	0.57 (0.21~1.09)	0.034

Data were expressed as median and ranges (minimum, maximum).

Abbreviations: IGF-1, insulin-like growth factor; IGFBP-3, IGF binding protein-3; GH, growth hormone

genotypic distributions of LEP-2548, LEPR K109R, and Q223R between the GHD subjects and normal controls (Table 2).

The minor allele (A allele) at LEPR Q223R was 7.4% in complete GHD subjects, while it was 36.4% in partial GHD subjects (*p* = 0.005). GHD subjects with the A allele (G/A or A/A, *n* = 26) at LEPR Q223R showed higher stimulated GH levels (*p* = 0.023) and lower height gain after 1 year of GH treatment (*p* = 0.034) than GHD subjects with the G allele (G/G, *n* = 67). GHD subjects with the G allele (A/G or G/G, *n* = 34) at LEP-2548 showed higher IGF-1 SDS (*p* = 0.047) and IGFBP-3 SDS (*p* = 0.027) levels than GHD subjects with the A allele (A/A, *n* = 59, Table 3). However, the IGF-1 SDS and IGFBP-3 SDS changes after 1 year of GH treatment were not relevant to the LEP-2548, LEPR K109R, and Q223R genotypes of the complete and partial GHD groups.

Discussion

In this study, the serum leptin levels and the distributions of three SNPs of leptin or leptin receptor did not differ between the GHD subjects and normal controls. However, higher levels of IGF-1 and IGFBP-3 in GHD subjects with the G allele at LEP-2548 than in subjects with the AA genotype may indicate the possibility of contributions to GHD pathogenesis. The lower height gains of the GHD subjects with the A allele at LEPR223

compared to subjects with the GG genotype after 1 year of GH treatment implies different effects of the genotype to the clinical course of the same disease entity; however, this topic needs to be researched in more detail.

The leptin gene is located on chromosome 7q32.1. Mutations in this gene and its regulatory regions cause extreme obesity in patients [25]. The specific promoter region of the leptin gene, LEP-2548 (rs7790039), is associated with obesity [26]. LEP-2548 is proximal to a binding site for the transcriptional factor Sp1 [27]. In terms of growth, the polymorphism of the LEP-2548 locus is associated with the serum levels of IGF-1 and leptin [28], which could affect GHD pathogenesis. In another study, the G allele of LEP-2548 was less frequent in GHD patients than in ISS patients [8]. In our study, the G allele of LEP-2548 in complete GHD subjects (25.9%) was less frequent than in partial GHD subjects (40.9%) or normal controls (30.4%); however, we did not detect statistical significance owing to the small number of subjects in the complete GHD group. In this context, GHD subjects with G allele at LEP-2548 showed higher serum levels of IGF-1 and IGFBP-3 than GHD subjects with the A allele, which may suggest a role of LEP-2548 in modulating growth potential *via* the IGF-1 pathway. However, the sex distribution, BMI status, body composition, or other clinical parameters according to LEP-2548 polymorphism are different between studies of growth or obesity [8, 29]. Therefore, many other factors related to leptin and leptin receptors are involved in

modulating the role of leptin in growth and obesity.

The leptin receptor gene is located on 1p31.3, and it is transcribed by 24 exons. Among several polymorphic loci in the LEPR gene, rs1137100 (K109R) and rs1137101 (Q223R) have been well studied in children with obesity and growth [9, 11, 28, 30]. Patients with the G allele had higher levels of insulin, leptin, and body fat percentage than patients with the A allele [31]. This finding was consistent with those of previous studies [32-35], which showed that having the G allele at rs1137101 (Q223R) incur an increased risk of being overweight, obese, and having related metabolic complications. In our study, the subjects with the G allele at Q223R were more prevalent in the complete GHD group than in the partial GHD group or normal controls, and the GG or GA genotypes showed different response to the growth hormone treatment compared to the AA genotype. There were differences in the BMI z-score between the complete and partial GHD and normal controls, which could be explained by the genotypic difference at LEPR223. The higher prevalence of the G allele at LEPR223 in the complete GHD subjects resulted in higher BMI z-scores in this group than in the partial GHD subjects or normal controls, in which the presence of the G allele at LEPR223 was less frequent, which was consistent with previous studies. Although the direct relationship between BMI z-score and genotype at LEPR223 was not significant, the role of the G allele at LEPR223 is expected to express obesity and affect the growth response to growth hormone in GHD subjects.

The rs1137100 (K109R), another leptin receptor polymorphic locus, has been examined in several obesity studies [30, 36]. We could not detect any significant genotypic differences at LEPR109 in our study, but the possible role of K109R in growth has been investigated previously [11]. Su *et al.* reported that there were differences in the genotypic distributions of polymorphism at

LEPR109 among the GHD, ISS, and normal control groups, and the frequency of the A minor allele was less lower in the GHD group [11]. The weight gain velocity before the GH treatment differed between the two groups depending on the genotype at LEPR109. However, these findings were significant only in male subjects; therefore, there could be a sex-dependent difference within the same disease.

This study had some limitations. First, the relatively small number of subjects with the complete GHD compared to the partial GHD or normal controls could have diminished the statistical power of the study. Partial GHD may have overlapping characteristics with ISS; therefore, it may not fully reflect the pathogenesis of GHD. Therefore, a sufficient number of complete GHD subjects may be needed to analyze the role of leptin/leptin receptor polymorphism in GHD pathogenesis. In line with this, no significant differences in the biochemical and molecular results between partial GHD and normal controls were detected. Second, body composition data such as fat mass and sex-stratified analysis were not available in this study. These confounding factors may affect leptin expression or serum leptin levels [37-39]; therefore, further investigations are needed.

There are ethnic differences in the linkage between SNP frequency and pathologic features [40, 41]. This study confirmed the associations between leptin/leptin receptor gene polymorphism and clinical/biochemical characteristics and the GH response in Korean GHD subjects. This may suggest the considerable role of leptin or leptin receptor gene as growth factors.

Conflict of Interest

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