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Identification of Novel Human *GH-1* Gene Polymorphisms that Are Associated with Growth Hormone Secretion and Height*

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ABSTRACT

Height, which is partially determined by GH secretion, is genetically influenced. The purpose of this study was to identify polymorphisms in the *GH-1* gene, which are associated with altered GH production. The subjects included prepubertal short children with GH insufficiency without gross pituitary abnormalities (n = 43), short children with normal GH secretion (n = 46), and normal adults (n = 294). A polymorphism in intron 4 (P-1, A or T at base 1663) was identified. Two additional polymorphic sites (P-2, T or G at base 218, and P-3, G or T at base 439) in the promoter region of the *GH-1* gene were also identified and matched with the P-1 polymorphism (A or T, respectively) in more than 90% of the subjects. P-1, P-2, and P-3 were considered to be associated with GH production, and the results of P-2 are explained as a representative in this abstract. For example, the allele frequency of T at P-2 in prepubertal short children with GH insufficiency without gross pituitary abnormalities (58.1%) was significantly different from that in short children with normal GH secretion and normal adults (37.0% and 43.5%, respectively; $P < 0.001$). Furthermore, significant differences were observed in maximal GH peaks in provocative tests (11.1 *vs.* 18.2 ng/mL, $P = 0.006$), insulin-like growth factor I SD scores (SDS) (−2.4 *vs.* −0.8, $P < 0.0001$), and height (Ht) SDS (−3.7 *vs.* −3.0, $P = 0/001$) in children with the T/T or G/G genotypes at P-2, respectively. In the entire study group, significant differences in insulin-like growth factor SDS (T/T, −0.9; G/G, −0.2; $P = 0.0009$) and Ht SDS (T/T, −1.0; G/G, −0.4; $P = 0.022$) were observed between the T/T and G/G genotypes at P-2. These data indicate that GH secretion is partially determined by polymorphisms in the *GH-1* gene, which explain some of the variations in GH secretion and Ht. (*J Clin Endocrinol Metab* **85:** 1290–1295, 2000)

HEIGHT (Ht) is determined by genetic, as well as environmental, factors. The involvement of genetic factors in Ht is supported by several reports about the significant correlation between final Ht and parental Ht and about the growth of twins (1–6).

The effect of GH secretion on Ht during childhood is well established. Patients with a deletion in the *GH-1* gene are extremely short (7–8). Ht during childhood correlates with GH secretion determined by measurements of either GH levels integrated over 24 h or serum GH-dependent parameters, such as insulin-like growth factor (IGF) I and IGF binding protein-3 (9–10).

Recently, in many endocrine disorders, polymorphisms of relevant human genes have been reported to be associated with polygenic disease. Examples of this includes the *β-3 adrenergic receptor* gene in noninsulin-dependent diabetes mellitus and obesity (11–13) and the *Vitamin D receptor* gene in osteoporosis (14).

We attempted to identify polymorphisms in the *GH-1* gene that are associated with GH production. A novel polymorphism in intron 4, which we called P-1, was verified to be associated with the GH secretion status. The close linkage of P-1 with two other polymorphic sites (P-2 and P-3) in the promoter region of *GH-1* was also shown.

Subjects

Three groups of Japanese subjects were studied (one adult and two pediatric cohorts). Children included prepubertal short children (Tanner stage = I, testis size < 3 cc) without any organic diseases whose height was less than −2 SD scores (SDS) (age, 4–11 yr; n = 89, male = 59) (15). The prepubertal short children were further subdivided into two groups, GH insufficiency (GHI) and normal short (NS) children. The definition of GHI and NS was based on the maximal and mean GH peaks of at least two GH provocative tests. The GHI group (n = 43, height, −3.51 ± 0.76 SDS at the time of diagnosis) had a mean GH peak of less than 10 ng/mL and

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a maximal GH peak of less than 15 ng/mL, without any abnormalities on magnetic resonance imaging (16–18) or other deficiencies of pituitary hormones. Eight of 43 GHI patients were considered to be patients with a severe type of GHI, since their maximal GH peaks were less than 5 ng/mL. In the NS group (n = 46, height, −3.05 ± 0.77 SDS), the mean GH peak was more than 10 ng/mL or the maximal GH peak was more than 15 ng/mL. Healthy normal Ht adults (NA) served as an additional control group (n = 294, male = 218; age, 22–45 yr; Ht SDS, 0.21 ± 0.95).

The prepubertal short children (GHI and NS) were younger than 10 yr for females and 11 yr, 6 months for males. The average age at the initial development of puberty in Japan is reported to be around 10- and 11.6-yr-old in females and males, respectively (19, 20). We limited the age at the time of the diagnosis of the GHI and NS groups because it is difficult to evaluate GH secretion at peripubertal periods, especially if puberty is delayed (21, 22). GH secretion is clearly increased during puberty, and short children with delayed puberty are reported to have transient decreases in GH reserve, when the reserve is determined by the results of standard GH provocative tests.

Materials and Methods

DNA was extracted from whole blood using standard techniques.

Confirmation of the sequence of the GH-1 *gene and restriction fragment length polymorphism (RFLP)*

Primers were designed for the reported sequence of the *GH-1* gene (23–25) to confirm the sequence, several of which were the same primers used by Igarashi *et al.* (26). The entire sequence of the *GH-1* gene, including about 300 bp of the promoter region, was determined in three NA subjects by direct sequencing of each of the PCR products in both directions.

RFLPs of the PCR products by the primers f1 and r1, which covered the entire coding region of the gene, were checked in 15 samples of the NA subjects. The enzymes used were Afa I, *Apa*I, *Bam*HI, EcoO 109 I, *Hin*fI, *Pst*I, *Sac*I, *Sac*II, and *Sma*I.

Identification of a polymorphism in intron 4

Intron 4 was screened for polymorphisms, since polymorphisms are commonly found in introns (14, 27, 28). PCR amplification of intron 4 of the *GH-1* gene was carried out with the flanking primers (f3: 5′-TGACTTTGAGAGCTGTGTTA-3′, and r1: 3′-ACAGACTGATCCA-CAGGAAGA-5′) (26), which generated a 542-bp product covering the entire sequences of intron 4. PCR conditions were: 95 C for 7 min, followed by 30 cycles of 95 C for 30 sec, 60 C for 30 sec, and 72 C for 90 sec, followed by a cycle with a 5-min extension at 72 C. PCR products showing a single band of the correct size were cleaved with *Hin*fI, which generated a 433- and 99-bp product (the longer fragment and the shorter fragment), followed by electrophoresis in 20% acrylamide/bis (49:1) gel with 10% glycerol after the step of denaturation with denaturing dye (95% formamide with 20 mmol/L EDTA and 10% glycerol) to detect polymorphisms of the *GH-1* gene. After this electrophoresis, DNA was visualized for the detection of single-strand conformation polymorphism (SSCP) by silver staining, as recommended by the manufacturer (Bio-Rad Laboratories, Inc.). To confirm the polymorphism(s), the direct sequence of the PCR product by a sequence analyzer (ABI PRISM TM 310, Genetic analyzer; Perkin-Elmer Corp.) was performed in both directions.

TABLE 1. Frequencies of the genotype and allele at P-1 and P-2

	P-1	P-2
	A/A A/T T/T	T/T T/G G/G
NA (n = 294)	62 124 108	64 128 102
	(A:T = 42.2:57.8)	(T:G = 43.5:56.5)
NS (n = 46)	7 19 20	8 18 20
	(A:T = 35.9:64.1)	(T:G = 37.0:63.0)
GHI (n = 43)	16 17 10	17 16 10
	(A:T = 57.0:43.0)[a]	(T:G = 58.1:41.9)[a]

[a] Represents a significant difference with either NA or NA ($P < 0.0001$).

The linkage of P-1 with polymorphic sites in other regions

After confirming the polymorphic site in intron 4 (P-1, detailed in *Results*), other introns, the 3-prime-untranslated region, the 5-prime-untranslated region, and the promoter region were similarly screened to see whether there were polymorphic sites that were linked with P-1. A previous report showed that an extensive polymorphism was generated in the 5′ untranslated region and the promoter region of the *GH-1* gene (29).

GH and IGF-I levels

GH peaks after standard GH provocative tests were measured by commercially available assays in 1985 and 1996 at each participating hospital. In more than 80% of the samples of the prepubertal short children, GH was analyzed by immunoradiometric assay (IRMA) (EIKEN, Tokyo, Japan) (30). GH levels measured by other assays were normalized to those of EIKEN IRMA based on the linear relationship between the EIKEN assay and the other assays (31). IGF-I was measured by IRMA in all the subjects (31–33) and was compared by using SDS. IGF-I is well known to be dependent on GH secretion (34–37).

Ht

Ht was measured every 3–4 months in the prepubertal children group (NS and GHI). Ht was checked by a questionnaire in adults (NA). Ht is expressed as SDS for the Japanese population (15).

Sample size calculations

After showing a difference in IGF-I SDS between genotypes, the necessary sample size to obtain a significant difference with a 95% confidence interval between genotypes was calculated.

Statistics and ethics

Statistical analysis was done using Statview version 4.5 and Statmate version 2.0. χ^2 analysis, and unpaired *t* test were used, and a *P* value less than 0.05 was considered to be significant.

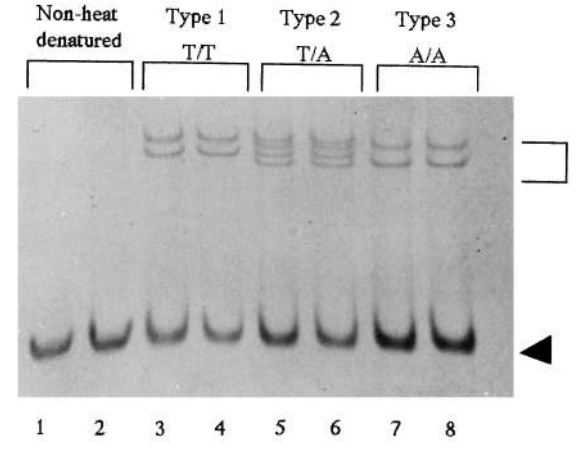

FIG. 1. SSCP gel of a part of intron 4 of the *GH-1* gene: types 1, 2, and 3 represent T/T, A/T, and A/A genotype, respectively, at P-1. Intron 4 is from base 1574–1826, and P-1 is at base 1663. An *asterisk* and an *arrow* represent the denatured bands of DNA and nondenatured bands of DNA, respectively.

The study was approved by the research board committee in the Tokyo Metropolitan Kiyose Children's Hospital.

Results

Confirmation of the sequence of the GH-1 *gene and absence of RFLPs*

The sequence of the *GH-1* gene was determined. There were at least 10 differences in the sequences from the papers reported (23–25). These differences were considered as either errors in the previous reports or polymorphic sites as written below. The sequences of the promoter region and of intron 4 are available, if requested.

No RFLPs were detected in the PCR products from the primers f1 and r1 by our methodology.

Identification of a novel polymorphism in the intron 4; P-1

The SSCP gel shown in Fig. 1 indicated the presence of a polymorphism (P-1) at base 1663 in intron 4; 1657-TAGCAGT/ACAGGCC-1669. The upper bands, which showed three different patterns (types 1, 2, and 3) reflect three types of the denatured strands of the shorter fragment of the PCR product. These three patterns were consistent with two different patterns of homozygous alleles (types 1 and 3) and one pattern of heterozygous alleles (type 2). Direct sequence analysis of the PCR products for both strands (25 samples) confirmed the data of the SSCP gel mentioned above, the representative data of which is shown in Fig. 2. Namely, it revealed that base 1663 in intron 4 had a transversion of T to A in either two alleles (type 3) or one allele (type 2). The sequence analysis of six samples after the subcloning the PCR products further confirmed the three types of base pairs combination at P-1 (data not shown).

Because there were no restriction enzymes that were useful for the identification of P-1, PCR, followed by SSCP, was done at least twice for the analysis of all samples.

Allele frequency of P-1 (Table 1)

The frequency of the genotype and allele at P-1 in each of three groups was determined, as shown in Table 1. The frequency of the A allele at P-1 in the GHI, NS, and NA groups was 57.0%, 35.9%, and 42.2%, respectively. The frequency of the polymorphism in the GHI group was significantly different from that in either the NS or NA group ($P < 0.001$ for both).

P-1 genotypes of the subjects with the severe type of GHI

Six of eight patients with the severe type of GHI had the A/A genotype at P-1, whereas two had the A/T genotype at P-1 (none had the T/T genotype). All the eight patients with the severe type of GHI showed normal sequence analysis of the PCR products by the combination of the primers f1 and r1, which covered all the exons and 300-bp promoter regions, including four introns (data not shown).

Maximal GH peaks, IGF-I SDS, and Ht SDS of the three genotypes at P-1

Table 2 shows the maximal GH peaks from GH provocative tests, the IGF-I SDS, and Ht SDS of the three genotypes

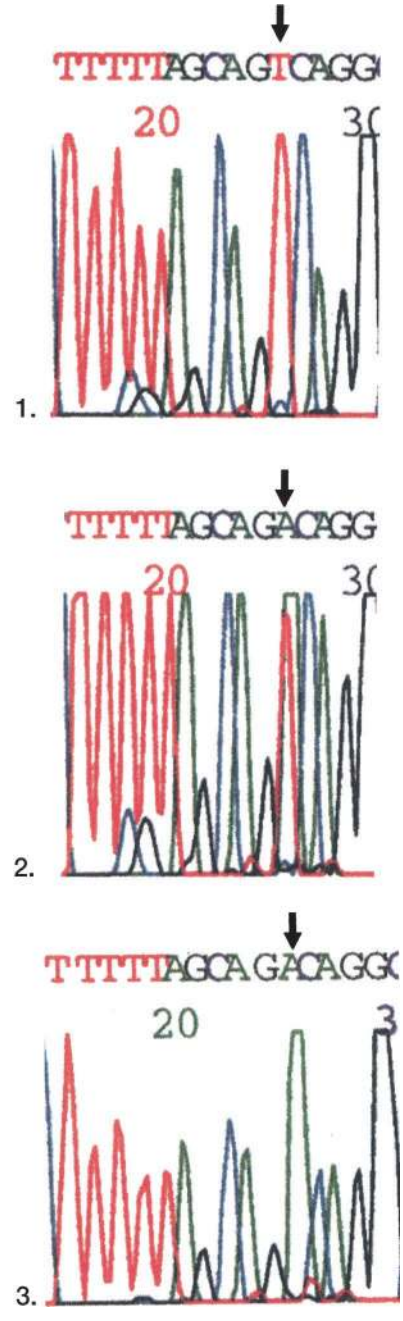

FIG. 2. Sequence of a part of intron 4 of the *GH-1* gene: 1, 2, and 3 correspond with types 1, 2, and 3 on the SSCP gel in Fig. 1, respectively.

at P-1. All three parameters were significantly different between the T/T and A/A genotypes at P-1 in prepubertal short children (GHI and NS); $P = 0.0012$ for GH peak, $P < 0.0001$ for IGF-I SDS, and $P = 0.0017$ for Ht SDS. In the adult group (NA), IGF-I SDS and Ht SDS were higher (with a trend toward significance) in the T/T genotype at P-1, compared with the A/A genotype at P-1 ($P = 0.14$ for IGF-I SDS, $P = 0.31$ for Ht SDS). This absence of a statistical significance was probably due to a much smaller sample size for the subjects with NA than the ideal one statistically calculated, which is shown in the last part of the *Results*. Inaccurate reported Ht

TABLE 2. Maximal GH peak (ng/mL), IGF-I (SDS), and Ht (SDS) of the three genotypes at P-1

	GH peak (ng/mL)	IGF-I (SDS)	Ht (SDS)
Group 1 (prepubertal; NS + GHI)			
A/A at P-1	10.3 ± 5.8 (23)	−2.32 ± 1.21 (23)	−3.58 ± 0.80 (23)
A/T at P-1	16.6 ± 12.1 (36)	−1.72 ± 1.29 (35)	−3.35 ± 0.86 (36)
T/T at P-1	18.2 ± 9.8 (29)	−0.83 ± 1.27 (30)	−2.95 ± 0.58 (30)
	$P = 0.0012$	$P < 0.0001$	$P = 0.0017$
Group 2 (adult; NA)			
A/A at P-1		−0.14 ± 1.00 (46)	0.15 ± 0.99 (62)
A/T at P-1		−0.04 ± 1.02 (102)	0.15 ± 0.94 (124)
T/T at P-1		0.12 ± 0.97 (86)	0.30 ± 0.93 (108)
		$P = 0.14$	$P = 0.31$
Group 1 + 2			
A/A at P-1		−0.87 ± 1.48 (69)	−0.86 ± 1.91 (85)
A/T at P-1		−0.47 ± 1.32 (137)	−0.62 ± 1.72 (159)
T/T at P-1		−0.12 ± 1.13 (116)	−0.40 ± 1.60 (138)
		$P = 0.0012$	$P = 0.075$

P values represent the difference between the A/A and T/T genotypes.
The numbers in *parentheses* show the numbers of the subjects studied.

TABLE 3. Maximal GH peak (ng/mL), IGF-I (SDS), and Ht (SDS) of the three genotypes at P-2

	GH peak (ng/mL)	IGF-I (SDS)	Height (SDS)
Group 1 (prepubertal; NS + GHI)			
T/T at P-2	11.1 ± 8.1 (25)	−2.35 ± 1.14 (25)	−3.67 ± 0.83 (25)
G/T at P-2	16.4 ± 11.6 (34)	−1.66 ± 1.32 (33)	−3.26 ± 0.81 (33)
G/G at P-2	18.2 ± 9.8 (29)	−0.83 ± 1.27 (30)	−2.95 ± 0.58 (30)
	$P = 0.0057$	$P < 0.0001$	$P = 0.004$
Group 2 (adult; NA)			
T/T at P-2		−0.14 ± 1.00 (48)	0.05 ± 0.97 (64)
G/T at P-2		0.02 ± 1.06 (104)	0.21 ± 0.95 (128)
G/G at P-2		0.06 ± 0.93 (82)	0.31 ± 0.93 (102)
		$P = 0.25$	$P = 0.086$
Group 1 + 2			
T/T at P-2		−0.90 ± 1.48 (73)	−0.99 ± 1.92 (89)
G/T at P-2		−0.39 ± 1.33 (137)	−0.51 ± 1.68 (161)
G/G at P-2		−0.18 ± 1.10 (112)	−0.43 ± 1.62 (132)
		$P = 0.0009$	$P = 0.022$

P values represent the difference between the A/A and T/T genotypes.
The numbers in *parentheses* show the numbers of the subjects studied.

(as compared to actual measured Ht) may also limit this observation. In the total subjects studied, IGF-I SDS and Ht SDS were higher in the T/T genotype at P-1 than the A/A genotype at P-1 ($P = 0.0012$ for IGF-I SDS, $P = 0.075$ for Ht SDS).

The possible linkage of P-1 with two additional polymorphic sites in the promoter region (P-2 and P-3)

No other polymorphic sites were found in introns 1, 2, 3, or the 3′ untranslated region by PCR-SSCP analysis, as described in *Materials and Methods*. The same methodology did not reveal a polymorphic site in the 5′ untranslated region either.

Two polymorphic sites in the promoter region (P-2, T or G at base 218; P-3, G or T at base 439) were identified; P-2 212-GCCTGCG/TGCCAGA-224, P-3 433-GTGGGGT/GCAACAG-445. These two polymorphic sites were linked with P-1 (A or T, respectively) in more than 90% of the subjects (see below). P-2 was suspected to be a polymorphic site, based on SSCP (Fig. 3) and was verified by direct sequence analysis of PCR products (n = 20, data not shown). There were no informative restriction enzymes available to detect the polymorphism at P-2. In Fig. 3, three types of patterns (types 4, 5, and 6) represent the T/T, T/G, and G/G genotypes at P-2, respectively. When the bases at the P-1 and P-2 sites were determined (in duplicates) by SSCP, 361 (93%) out of the total 388 samples were matched, which means that A or T at P-1 was matched with T or G at P-2, respectively. In 12 of the 27 samples that were discordant between the results of P-1 and P-2 (such as A/T genotype at P-1 and G/G genotype at P-2), the discordance was verified by direct sequencing of the respective PCR products (data not shown).

P-2 was associated with the GH secretion status similarly to P-1. First, the allele frequency of P-2 genotypes in the GHI group was significantly different from that in the NA and NS groups (Table 1). Second, six of the eight patients with severe GHI had the T/T genotype at P-2 and the other two had the T/G genotype at P-2. Third, peak GH levels, IGF-I SDS, and Ht SDS, as defined above in the prepubertal short children groups (NS + GHI) and in the total subjects studied, were significantly higher in the G/G genotype at P-2 compared to the T/T genotype at P-2 (Table 3).

The P-3 polymorphism was verified by direct sequencing (n = 14) of the relevant PCR products and the presence of the T or G base at P-2 matched in all cases with the presence of

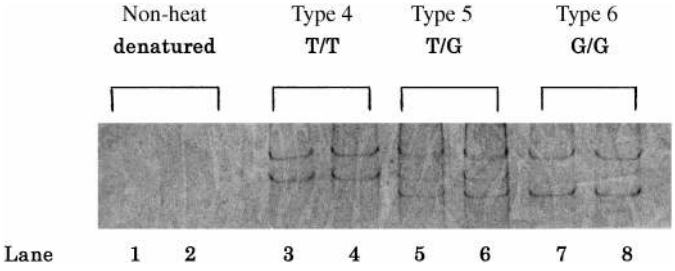

FIG. 3. SSCP gel of a part of the promoter region of the *GH-1* gene: types 4, 5, and 6 represent T/T, T/G, and G/G genotypes, respectively, at P-2. The transcription starting site is base 517, and P-2 and -3 are bases 218 and 439.

the G or T base at P-3 (data not shown). P-3 was difficult to detect by the PCR and SSCP methods.

No complete match with published response elements of known transcriptional regulators were found at or around either P-1, P-2, or P-3 by searching the genebank data base (Genome Data Base).

Sample size calculations

Since the difference between the IGF-I SDS among the two genotypes in the NA group was not significant, we estimated the required sample size using the data of Tables 1 and 3. The sample size required to detect a significant difference in IGF SDS between these two genotypes at P-1 and P-2 was calculated to be 600-1300 for $\alpha = 0.05$ and power, 0.85–0.95. The actual sample numbers of the A/A and T/T genotypes at P-1 were 125, which is well below the ideal numbers, as calculated above.

The ideal sample sizes of the prepubertal short children groups (NS + GHI) required to detect a significant difference in IGF SDS between these two genotypes at P-1 and P-2 were similarly calculated to be 26–36 for subjects with A/A and T/T genotype at P-1 or T/T and G/G genotype at P-2, which is less than the actual number of these two genotypes (n = 55).

The ideal sample sizes of total subjects required to detect a significant difference in IGF SDS between the two genotypes at P-1 and P-2 were similarly calculated to be 54–76 for subjects with A/A and T/T genotype at P-1 or T/T and G/G genotype at P-2, which is less than the actual number of these two genotypes (n = 185).

Discussion

Three polymorphisms in the *GH-1* gene were identified in this study, which may explain part of the genetic determination of GH secretion at the molecular level, at least in Japanese. Polymorphisms in the *GH-1* gene may also be associated with idiopathic GH deficiency. The molecular mechanism of variation in GH secretion has not yet been fully analyzed. Although more than 10 polymorphic sites of the *GH-1* gene were reported (24, 25, 29, 38), none of them were shown to be associated with GH secretion status. P-1 has not been reported so far, to our knowledge. P-2 and P-3 were previously reported but not thought to be associated with the GH secretion status (29, 38).

One of the advantages of this study is that total GH production was evaluated directly and quantitatively by stimulated GH levels and basal IGF-I levels, which are well documented to be *GH-1* dependent. On the other hand, in most other reported polymorphisms, such as the polymorphism of the *β3-adrenergic receptor* gene (11–13) and the *IRS-1* gene (39–40), the amount of the expressed protein was not estimated directly.

It remains to be determined which polymorphic site in the *GH-1* gene is directly related to the amount of the total GH mRNA expression from the *GH-1* gene. First, a direct transcriptional effect of P-1 or some other sequence of the intron, which is linked with P-1 and was not found in this study, is possible (41, 42). Second, P-2, P-3, or other sites in the promoter region of the *GH-1* gene may directly regulate *GH-1* transcription as previously speculated for other genes (43–46). Third, it is possible that a sequence in one of the *GH-1* exons, which is linked with either P-1, P-2, or P-3, is related with the amount of the total GH mRNA expression from the *GH-1* gene, as described in other genes (11–13, 39–40, 47). A point mutation of the coding region of the *GH-1* gene was reported to cause the absence or decrease in GH secretion (48–52), which allows for the hypothesis that the polymorphisms of exons of the *GH-1* gene may be considered as part of the continuous spectrum of mutations in this gene. The study of the molecular mechanisms of this phenomenon would be worth pursuing.

Contributions of mechanisms other than the polymorphisms in the *GH-1* gene reported here accounting for variation in GH secretion can not be excluded. Polymorphic site(s) in the *GH-1* gene other than P-1, -2, and -3 that were not identified in this study may also be associated with GH secretion in an additive way. Polymorphic site(s) in the gene(s) encoding other hormonal factors influencing GH production might also be related to GH secretion. Some of these have never been analyzed; for example, polymorphic site(s) in the *GRF*, *somatostatine*, *GRF receptor*, and *somatostatine receptor* genes may be related to GH production.

Ht is determined by various genetic and environmental factors. Given that Ht is partially determined by GH secretion (7–10), differences in Ht SDS observed in our prepubertal group (NS + GHI), as well as the total subjects in this report, suggest that variation in Ht can also be explained by polymorphisms of the *GH-1* gene. Further study based on large numbers of healthy subjects with measured Ht data, which would be ideally homogeneous in every respect, including age, is needed to explore the extent to which of these *GH-1* gene polymorphisms contribute to the individual variation of Ht.

In conclusion, polymorphisms in the *GH-1* gene were identified, and their association with GH secretion and Ht were shown. This is a major step toward understanding the mechanisms accounting for variation in GH secretion and Ht. Further study is needed to validate the mechanisms.

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