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Genetic Polymorphisms in the Locus Control Region and Promoter of *GH1* Are Related to Serum IGF-I Levels and Height in Patients with Isolated Growth Hormone Deficiency and Healthy Controls

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Key Words

Growth hormone · Locus control region · Promoter · Single nucleotide polymorphism · Insulin-like growth factor 1

Abstract

Background/Aims: Expression of the human growth hormone (GH) gene (*GH1*) is regulated by a locus control region (LCR) and the highly polymorphic *GH1* promoter. We analyzed *GH1* LCR/promoter single nucleotide polymorphisms (SNPs) in patients with isolated growth hormone deficiency (IGHD) in relation to clinical data. **Methods:** We directly sequenced the *GH1* LCR/promoter of 62 Dutch IGHD patients without mutations or deletions in *GH1* or *GHRHR* and of 72 controls with normal height. We related *GH1* LCR/promoter SNPs to height, serum insulin-like growth factor 1 (IGF-I) levels and response to GH treatment. **Results:** In IGHD patients, promoter SNPs 6, 8 and 9 were associated with height and IGF-1 levels. In controls, SNPs 6 and 11 were associated with height. Homozygosity for the minor allele of SNP 9, associated with lower IGF-I levels in patients, was significantly more frequent among patients than among controls. Genotypes based on SNPs 6, 8, 9 and 11 explained 10.8% of IGF-I

SDS variation in IGHD patients and 15.9% of height SDS variation in controls. **Conclusion:** *GH1* Promoter SNPs 6, 8 and 9 were associated with height and IGF-1 levels among patients, and SNPs 6 and 11 with height in controls.

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Introduction

Human postnatal growth is determined by the interaction of various genetic and environmental factors, and results from the lengthening of bones via cellular divisions, which is mainly regulated by human growth hormone (GH). GH, secreted by the somatotrophs of the anterior pituitary gland, is a protein of 191 amino-acids (22 kDa) with 2 disulfide bridges, which are important for its structure and bioactivity. GH synthesis and secretion are regulated by several hormones, reviewed by Goldenberg and Barkan [1]. GH releasing hormone is produced in the hypothalamus and stimulates GH production via binding to the GHRH receptor (GHRHR), which is located in the anterior pituitary. GH production is negatively regulated by the somatotropin release inhibiting factor, or so-

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matostatin, and by insulin-like growth factor 1 (IGF-I), the end product of GH's action.

Mutations in the genes encoding GH and GHRHR (*GHI* and *GHRHR*, respectively) can cause isolated growth hormone deficiency (IGHD) types IA, IB (OMIM No. 262400) and II (OMIM No. 173100). The frequency of *GHI* and *GHRHR* mutations in IGHD patients varies between 0 and 32% among different countries [2–7]. Thus, in the best of cases, mutations in the coding regions of *GHI* and *GHRHR* are only detected in one third of the IGHD patients.

GHI is located in the human GH cluster, in which 5 genes are situated on chromosome 17. Human GH (*GHI*) lies at the 5' end of the cluster and is mainly expressed in pituitary somatotrophs. The remaining 4 genes, placental growth hormone (*GH2*) and 3 chorionic somatomammotropins (*CSI*, *CS2*, and the pseudogene *CS5* or *CSHP1*), are only expressed in the placenta.

The expression of *GHI* is regulated by a locus control region (LCR), located 14.5–32 kb upstream of the gene [8] and by the *GHI* promoter. To date, 3 single nucleotide polymorphisms (SNPs) have been reported in the LCR [9], while the *GHI* promoter is highly polymorphic [9–14].

Although, in general, SNPs do not necessarily cause disease, they have been associated with susceptibility to diseases [15–17] and to environmental toxins [18] by affecting splicing [19], allelic expression [20], nonsense-mediated mRNA decay [21] or transcription factor binding [22]. *GHI* promoter SNPs have been associated with height [9, 10, 12], bone density, bone loss and fetal growth restriction [23, 24]. In addition, some *GHI* promoter SNPs have been associated with breast and colorectal cancer [13, 25–28]. Two promoter SNPs have been studied in relation to GH and/or IGF-I levels in patients with varying degrees of GH deficiency [11, 12].

The Dutch Hypothalamic and Pituitary Gene Study is a nation-wide multicenter study investigating genetic and other causes of 'idiopathic' growth hormone deficiency in the Dutch GH deficiency population. In order to explain the variability in our IGHD patients' phenotypes and their variable response to GH treatment, we analyzed *GHI* LCR/promoter sequence variations in IGHD patients and looked for associations of these variations with height, serum IGF-I levels and height increases during the 1st year of GH treatment. We compared the data with those of a healthy Dutch control group. Since Hasegawa et al. [12] related the SNP 'P1' in *GHI* intron 4 (dbSNP 2665802) with height, GH and IGF-I levels, we also included this polymorphism in our analysis.

Patients and Methods

We included 62 Dutch Caucasian patients diagnosed with IGHD based on a scoring system including height SDS (HSDS), maximum GH levels, IGF-I and IGFBP-3 SDS without mutations or deletions in *GHI* or *GHRHR* [29]. Patients' clinical data were available from the Dutch National Registry of Growth Hormone Treatment, where auxologic and laboratory parameters have been documented.

GH and IGF-I measurements were centrally performed in 1 laboratory with published reference values [30] for 80% of the patients. For the remaining 20%, the laboratory-specific reference values for IGF-I were used to calculate the SDS. We obtained approval from the medical ethics committees of all participating hospitals. Informed consent was obtained from all participating patients or their parents, if the patients were aged less than 18 years. Seventy-two healthy Caucasian Dutch young adults with a height SDS between –2 and +2 agreed to participate in DNA analysis as control subjects.

Genomic DNA, extracted from peripheral blood according to standard procedures, was used for direct sequencing of the *GHI* LCR/promoter using an ABI 3100 Sequencer. A 1,231-bp fragment including the promoter of *GHI* was amplified using forward primer 5'-GGGAGCCCCAGCAATGC-3' and reverse primer 5'-TCTGCCTGCATTTTCGCTTCG-3' by Touchdown PCR, including denaturation for 10 min at 95°C, amplification at 68–61°C decreasing the annealing temperature by 1°C every 2nd cycle and 25 subsequent cycles at 61°C, followed by a 7-min extension at 72°C. This fragment was sequenced by forward primers 5'-GGGAGCCCCAGCAATGC-3' and 5'-CTGTCTGGTGGGTG-GAGGTTAAA-3', and reverse primers 5'-CACATTCAGAAG-CCCCAAAC-3' and 5'-ACCCAACCTGTCTCTCTTTA-3'.

Since Hasegawa et al. [12] related the SNP 'P1' (IVS4 +90 T/A, dbSNP 2665802) in *GHI* intron 4 with height, GH and IGF-I levels, we included this polymorphism in our analysis. P1 genotypes were available for patients who had participated in the genetic screening of the coding regions of *GHI* and *GHRHR* as part of the Hypothalamic and Pituitary Gene Study [29]. In these patients, exon 4 and intron 4 had been PCR-amplified with forward primer 5'-CCGT-GAGTGGATGCCTTCTC-3' and reverse primer 5'-GTGAGT-TCTCTTGGGTCAGG-3' (annealing temperature 57.7°C), and the fragment had been screened by WAVE dHPLC at 62.1 and 62.6°C, which were shown to accurately detect the P1 variant [29].

We used the *GHI* gene nucleotide sequence obtained from GenBank (Accession No. J03071) as a control and we numbered the SNPs according to Horan et al. [9]. We obtained the reference LCR sequence from GenBank (Accession No. AF010280) and used LCR numbering according to Jin et al. [31]. Of the LCR/promoter SNPs, linkage disequilibrium represented by R^2 and D' was analyzed using Haploview [32].

The associations between genetic and clinical parameters were analyzed by means of ANOVA and χ^2 tests using SPSS Version 11.0. Response to GH treatment was defined as the increase in HSDS during the 1st year of GH treatment. The normality of the distribution of all analyzed parameters was assessed with the Kolmogorov-Smirnov and Shapiro-Wilk tests. When expected cell counts for comparison of genotype frequencies between patients and controls were below 5, we used Fisher's exact test instead of the χ^2 test. Parameters that were not normally distributed were analyzed using the nonparametric Kruskal-Wallis test instead of ANOVA.

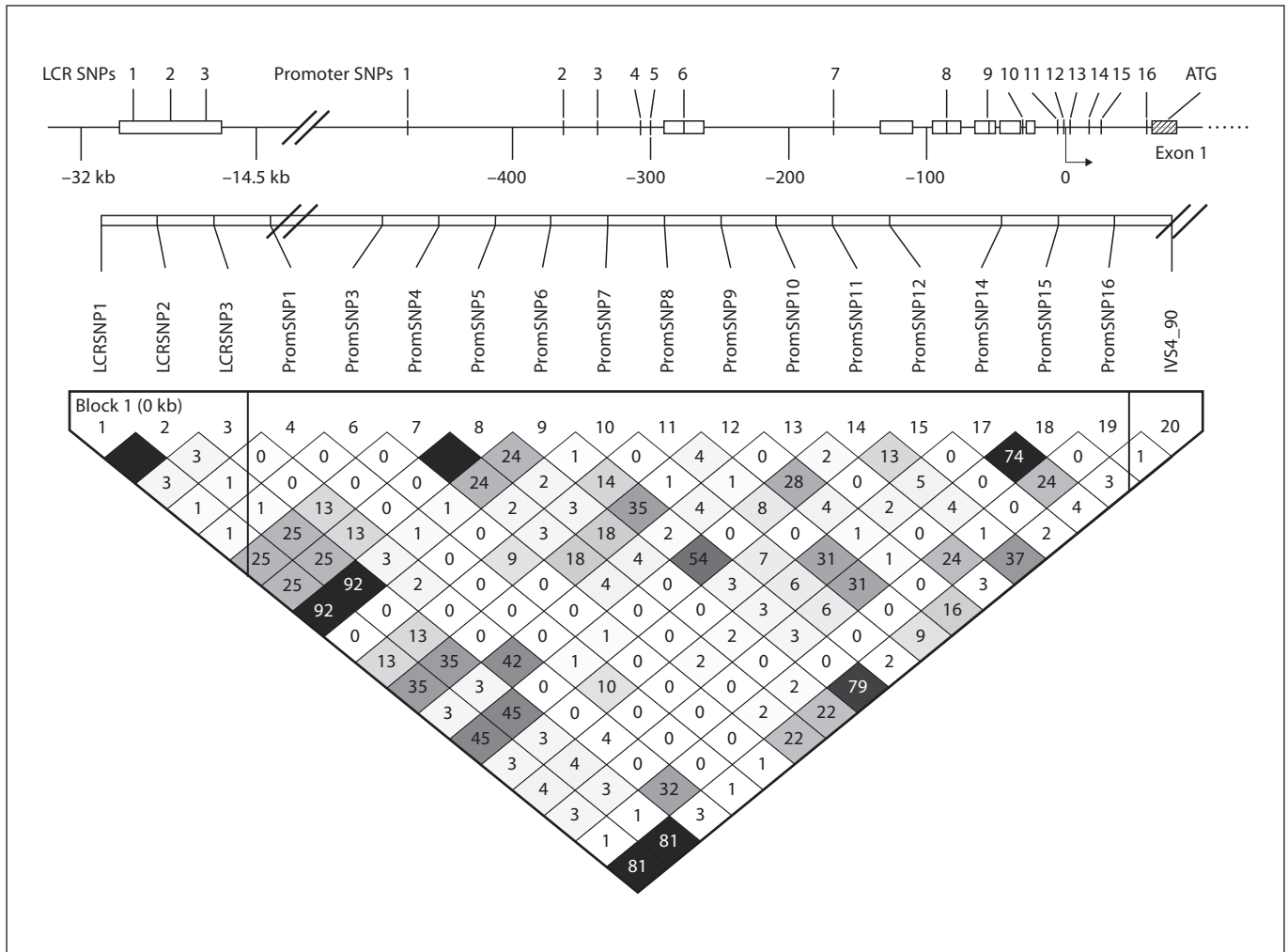


Fig. 1. LD plot showing linkage disequilibrium between *GHI* LCR, promoter and intronic SNPs. Black squares indicate SNPs that are in 100% LD ('highest possible correlation') with other SNPs. Dark and light gray squares represent lower levels of linkage disequilibrium, represented by R^2 values in the squares. In the schematic representation of the *GHI* LCR and promoter, open

boxes represent binding sites (for PIT1, nuclear factor 1, vitamin D response element) and the TATA box, the hatched box with ATG represents the transcriptional initiation codon, and numbers represent SNP numbers. Prom = Promoter; IVS4_90 = intronic SNP 'P1' IVS4 +90 T/A; 0 = 1st base of *GHI* cDNA sequence.

Results

All LCR/promoter SNPs conformed with the Hardy-Weinberg equilibrium according to a χ^2 test ($p > 0.05$) except for promoter SNP 8 ($p = 0.01$). In contrast to Horan et al. [9], we found that promoter SNP 2 was not polymorphic since all patients and controls carried the same allele. In patients and controls, there was a 100% linkage disequilibrium between LCR SNP 1 and 2, and there was a 100% linkage disequilibrium between promoter SNPs 4 and 5. Gene structure, sequence variation and linkage disequilibrium between LCR and promoter SNPs in our

patients is shown in figure 1. In patients and controls, promoter SNP 6 was strongly linked with LCR SNP1 (D' 0.96, R^2 0.92; fig. 1). In patients, promoter SNP 6 was strongly linked with SNP 'P1' in intron 4 of *GHI* (D' 0.95, R^2 0.72; P1 genotypes were not available for controls, see methods section).

Height in cases and controls, and serum IGF-I levels in patients were normally distributed. Four promoter SNPs were related with height and IGF-I levels. An overview of these SNPs, their rs numbers, alleles and their relations with clinical parameters are shown in tables 1 and 2, as well as figure 2.

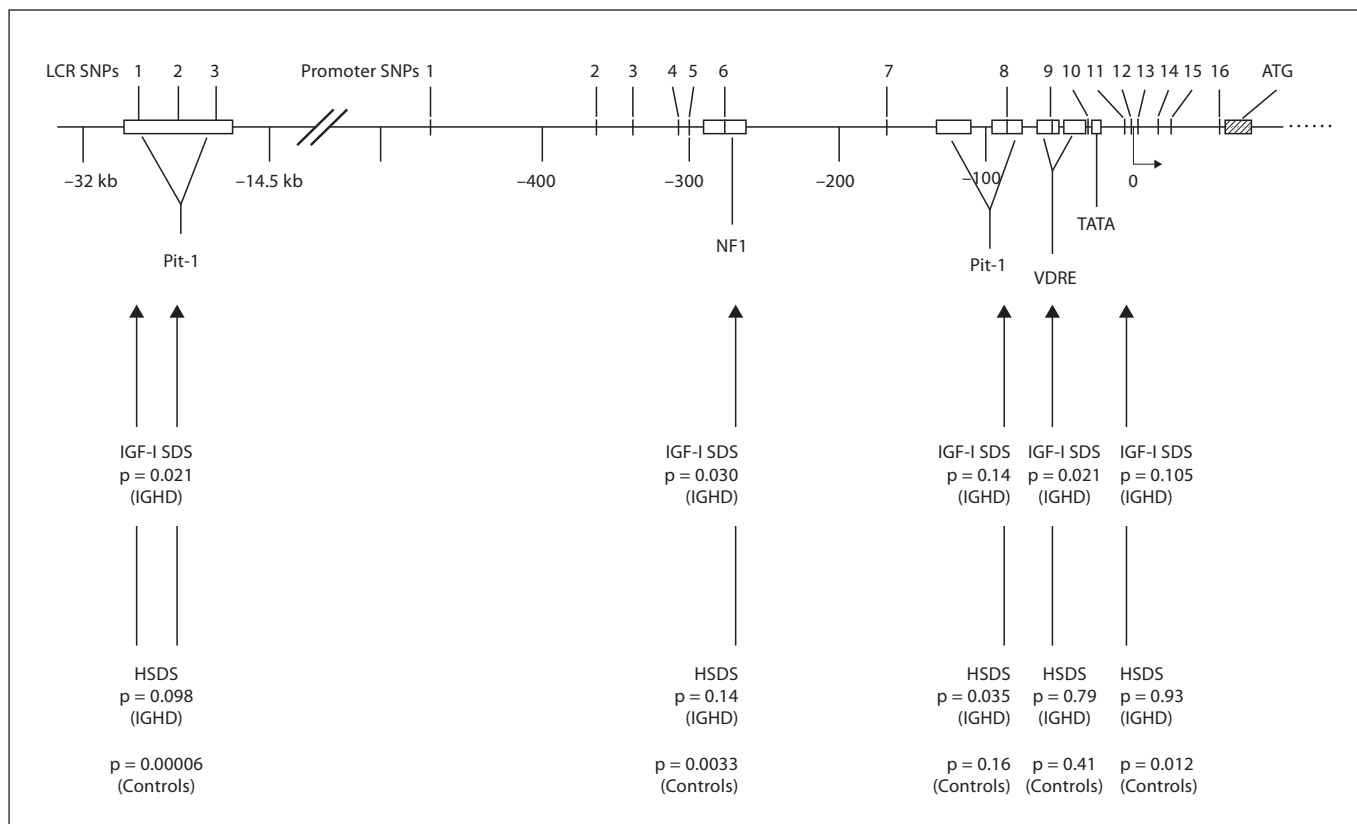


Fig. 2. *GH1* promoter and LCR SNPs genotyped in this study, the functional regions in which they are located and their associations with clinical data (adapted from Horan et al. [9]). NF1 = Nuclear factor 1; VDRE = vitamin D response element.

Since genotype frequencies and clinical associations differed between IGHD patients and controls, we discuss the results of the IGHD patients and healthy controls separately.

IGHD Patients

The study population consisted of 43 male and 19 female IGHD patients, which is in accordance with a male predominance in the overall Dutch IGHD population. Mean (\pm SD) age at testing was 15.6 ± 6.4 years, mean age at the start of GH treatment was 5.7 ± 2.7 years. At the start of GH treatment, height SDS was -3.1 ± 0.9 and IGF-I SDS was -3.3 ± 2.3 . There were no differences in genotype frequencies, height SDS or IGF-I SDS between males and females.

We found significant differences in height and serum IGF-I levels between carriers of the different genotypes at individual promoter SNPs 6, 8 and 9 (table 2). LCR SNP1, promoter SNP 6 and intron 4 SNP 'P1' (IVS4 +90 T/A) were highly linked (fig. 1). In our population, there was

no significant association between the individual intron 4 SNP and peak GH levels, HSDS and IGF-I levels as formerly described by Hasegawa et al. [12] (data not shown).

We did not find any relation between LCR/promoter SNPs and the 1st year response to GH treatment (data not shown).

Controls

The controls (27 males and 45 females) had a mean (\pm SD) age at testing of 21.0 ± 1.6 years and height SDS of -0.2 ± 1.0 . There were no differences in genotype frequencies or height SDS between males and females.

For promoter SNPs 6 and 11, height differed significantly between the carriers of the various genotypes (table 2).

IGHD Patients versus Controls

We compared promoter SNP genotypes between IGHD patients and controls, and found that homozygous

Table 1. SNPs located in the LCR, promoter and intron 4 of *GH1*

SNP ID ^a	Position ^b	Reference	<i>GH1</i> allele ^c	Functional region
LCR SNP 1/2 ^e	1194/1144	rs4968672/rs4968673	G/A ^f /T ^f /G ^f	PIT1
LCR SNP 3	990	–	C/T ^f	PIT1
Prom SNP 1	–476	rs41299065	G/A ^f	
Prom SNP 2	–364	–	G ^d	
Prom SNP 3	–339	rs62636320	G/– ^f	
Prom SNP 4/5 ^e	–308/–301	rs1811081/rs2011732	G/T ^f	
Prom SNP 6	–278	rs2005171	G/T ^f	NF1
Prom SNP 7	–168	rs2727338	T/C ^f	
Prom SNP 8	–75	rs11568828	A/G ^f	PIT1
Prom SNP 9	–57	rs2005172	G/T ^f	VDR
Prom SNP 10	–31	rs11568827	G/– ^f	TATA
Prom SNP 11	–6	rs6171	A/G ^f	TSS
Prom SNP 12	–1	rs695	A/T ^f /C ^f	TSS
Prom SNP 13	+3	rs6175	G/C ^f	TSS
Prom SNP 14	+16	rs9282699	A/G ^f	5'-UTR
Prom SNP 15	+25	rs6172	A/C ^f	5'-UTR
Prom SNP 16	+59	rs6173	T/G ^f	5'-UTR
<i>GH1</i> SNP P1	IVS4+90	dbSNP 2665802	T/A ^f	Intron 4

TSS = Transcriptional start site; PIT1 = PIT1 binding site; NF1 = nuclear factor 1 binding site; VDR= vitamin D receptor binding site; Prom = promoter; – = not available.

^a SNP identification according to Horan et al. [9]. ^b SNP position relative to *GH1* transcriptional start site. ^c The most frequent genotypes in our study at the various promoter SNP sites are in accordance with the expected genotype when sequencing *GH1* instead of any of the other four *GH1* paralogue genes. ^d Only G alleles were reported by Horan et al. [9] and our study. ^e SNPs are 100% in linkage disequilibrium with each other. ^f Minor allele.

Table 2. Frequencies of individual promoter SNPs and their relations with clinical data

		IGHD patients			Controls	
		n	HSDS	IGF-I SDS	n	HSDS
Promoter SNP 6	GG	22 (37%)	–3.3 (0.8)	–4.0 (2.6)	19 (27%)	0.3 (0.7)
	GT	32 (53%)	–3.1 (0.9)	–3.0 (2.0)	42 (59%)	–0.6 (0.9)
	TT	6 (10%)	–2.5 (0.8)	–1.4 (1.2)	10 (14%)	–0.1 (0.8)
		T = 37%	p = 0.14	p = 0.03	T = 44%	p = 0.003
Promoter SNP 8	AA	53 (88%)	–3.2 (0.9)	–3.4 (2.3)	63 (87%)	–0.3 (0.9)
	AG	7 (12%)	–2.5 (0.7)	–2.0 (1.0)	7 (10%)	–0.6 (0.8)
	GG	0	–	–	2 (3%)	0.8 (1.2)
		G = 6%	p = 0.04	p = 0.14	G = 8%	p = 0.16
Promoter SNP 9	GG	25 (42%)	–3.1 (0.8)	–2.6 (1.6)	25 (35%)	–0.2 (0.9)
	GT	22 (37%)	–3.1 (1.1)	–3.0 (2.3)	42 (58%)	–0.4 (1.0)
	TT	13 (22%)**	–3.3 (0.8)	–4.8 (2.6)	5 (7%)	0.2 (0.7)
		T = 40%	p = 0.8	p = 0.02	T = 36%	p = 0.41
Promoter SNP 11	AA	24 (40%)	–3.2 (0.7)	–4.0 (2.5)	18 (25%)	0.0 (1.0)
	AG	31 (52%)	–3.1 (1.0)	–2.9 (2.0)	43 (60%)	–0.6 (0.9)
	GG	5 (8%)	–3.2 (0.7)	–2.0 (1.6)	11 (15%)	0.2 (0.9)
		G = 34%	p = 0.9	p = 0.10	G = 45%	p = 0.012

HSDS = height SDS (for patients at start of GH treatment).

** Homozygosity for the minor allele is more frequent in IGHD patients than in controls, p = 0.005.

Table 3. Frequencies of combined genotypes of promoter SNPs 6, 8, 9 and 11 and their relations with clinical data

SNP 6-8-9-11 genotype*	IGHD patients							Controls		
	n ¹	%	height SDS	peak GH ⁴ arginine (mU/l)	peak GH ⁴ clonidine (mU/l)	IGF-I ² SDS	n ¹	%	height ³ SDS	
1 G/G A/A T/T A/A	12	27.2	-3.2 (0.8)	12.1 (9.2)	9.5 (5.1)	-5.0 (2.6)	4	8.3	0.4 (0.5)	
2 G/G A/A G/T A/A	5	11.4	-3.5 (0.8)	17.6 (1.3)	15.0 (7.2)	-2.1 (2.3)	6	12.5	0.5 (0.8)	
3 G/G A/A G/T A/G	0	-	-	-	-	-	6	12.5	0.0 (0.9)	
4 G/T A/A G/T A/G	12	27.2	-3.2 (1.3)	11.0 (5.1)	12.0 (5.2)	-3.7 (2.6)	26	54.2	-0.6 (0.9)	
5 G/T A/A G/G A/G	9	20.5	-3.2 (0.8)	9.0 (5.8)	11.5 (5.2)	-2.7 (1.2)	5	10.4	-0.5 (0.8)	
6 G/T A/G G/G A/G	3	6.8	-3.0 (0.1)	6.5 (5.6)	12.0 (0.1)	-2.2 (1.7)	1	2.1	-0.4	
7 T/T A/A G/G A/G	3	6.8	-3.0 (0.6)	9.3 (5.2)	10.6 (6.9)	-1.3 (0.7)	0	-	-	
	44	100.0					48	100.0		

Data are shown as mean (SD).

* Only genotypes present in at least 5% of the patients or controls are shown.

¹ Difference in genotype frequencies between IGHD patients and controls; $p = 0.002$.

² IGF-I SDS difference between genotypes; $p = 0.08$.

³ Height SDS difference between genotypes; $p = 0.05$.

⁴ Peak GH levels did not differ significantly between genotypes (tested by nonparametric Kruskal-Wallis test due to non-normal distribution of the parameters).

ity for the minor allele of SNP 9 was more frequent among patients than among controls (21% vs. 7%, $p = 0.005$, table 2). Since the minor allele at SNP 9 is associated with lower IGF-I levels in patients, this suggests this allele might contribute to impaired GH levels in IGHD patients.

The promoter SNP 12 was tri-allelic with the major allele A occurring in controls in combination with 2 different minor alleles (AC and AT, frequencies AA 74%/AT 19%/AC 5%/TT 1%), whereas in patients, only the AT genotype was present. Among controls, there was no difference in height SDS between individuals with the AC and those with the AT genotype.

For promoter SNP 13, the minor allele was present in 4 of 72 controls (6%), whereas all 62 patients were homozygous for the major allele ($p = 0.124$). For the remaining SNPs, genotype and allele frequencies were similar for patients and controls.

Multiple Regression

The promoter SNPs that were associated with height and/or IGF-I levels in patients or controls (SNPs 6, 8, 9 and 11) were present in 23 different combinations ('SNP 6-8-9-11 genotypes'), of which 7 were present in at least 5% of the patients or controls (table 3). Only 48 controls and 44 patients carried these 7 most frequent combined genotypes; clinical data of the patients and controls with these genotypes are shown in table 3.

Backward multiple regression showed that the 'SNP 6-8-9-11 genotypes' (numbered 1-7 according to table 3) explained 10.8% of the variation in IGF-I SDS in IGHD patients (adjusted R^2 0.108, $p = 0.02$). In controls, the 'SNP 6-8-9-11 genotypes' explained 15.9% of HSDS variation (R^2 0.159, $p = 0.003$).

Discussion

We analyzed *GHI* LCR/promoter SNPs in IGHD patients without mutations or deletions in *GHI* or *GHRHR*, and examined whether these SNPs were associated with height, serum IGF-I levels and response to GH treatment in order to explain the phenotypic variability present among IGHD patients. We compared patients' data with those of a normal control group and found that homozygosity for the minor allele of SNP 9 was more frequent among patients than among controls (21% vs. 7%, $p = 0.005$). The minor allele at SNP 9 was associated with lower IGF-I levels in patients, suggesting that this allele might contribute to impaired GH production in IGHD patients. Also SNP 6, 8 and 11 were related with height and IGF-I levels.

LCR SNPs 1 and 2, promoter SNP 6 and intron 4 SNP 'P1' (IVS4 +90 T/A; only available for IGHD patients) were highly linked. However, in contrast to Hasegawa et al. [12], we did not find any significant association between the individual intron 4 SNP and peak GH levels, HSDS,

and IGF-I levels in our population (data not shown), whereas we did find significant associations between LCR SNPs 1 and 2 and growth-related parameters, as well as between promoter SNP 6 and these parameters (fig. 2). This suggests that the relation between the intron 4 SNP and growth-related parameters reported by Hasegawa et al. [12], might actually be caused by its linkage with LCR SNPs 1 and 2 or promoter SNP 6, which, in our study, have stronger associations with growth-related parameters, but were not studied by Hasegawa et al. [12].

We tried to combine promoter SNPs 6, 8, 9 and 11 into haplotypes. Although the result of the multiple regression based on these combined genotypes should be interpreted with caution due to the reduced patient number and subsequent power reduction, in our population the combined genotypes explained 10.8% of the variation in serum IGF-I levels in IGHD patients and 15.9% of height variation in controls. Although all patients had IGHD according to Dutch consensus criteria, certain genotypes were associated with a relatively mild phenotype.

The impact of individual SNPs can be explained by the fact that the regions in which they are located are important for transcription of *GHI*. The LCR contains DNase I-hypersensitive sites located at -14.5 to -32 kb relative upstream to the *GHI* promoter, where the transcription factor PIT1 binds in order to regulate *GHI* transcription. Therefore, LCR SNPs may alter transcription of *GHI*. SNP 6 is located in the part of the promoter where nuclear factor 1 binds [33]. Binding of nuclear factor 1 to the nuclear factor 1 binding site is necessary for transcription, and variation at this site may alter expression of *GHI*. Promoter SNP 8 is located in the proximal PIT1 binding site and promoter SNP 9 is located in the region corresponding to the vitamin D receptor response element. Disturbed binding of PIT1 and the vitamin D receptor to the PIT1 binding site and vitamin D receptor response element, respectively, may alter transcription and, thus, expression of the *GHI* gene. Both SNP 8 and 9 were shown in vitro to interact with nucleic acid binding proteins, and alternative alleles exhibit differential protein binding [9]; SNP 9 showed protein interaction only for the G allele, not the T allele [9]. This is in accordance with our findings in vivo that patients homozygous for the T allele have very low IGF-I levels (table 2). For SNP 8, Esteban et al. [10] demonstrated a lower HSDS in carriers of the alternate allele in a control normal height adult population; Giordano demonstrated a diminished transcriptional activity for the alternate allele [11]. For SNP 9, Giordano demonstrated diminished GH secretion in alternate allele carriers

[34]. SNP 11 is located in the transcriptional start site of *GHI*; therefore, variation at this site may alter efficacy of transcription, leading to varying levels of *GHI* expression. Probably, the effect of the promoter SNPs is the sum of individual SNP effects and effects based on combinations with other SNPs. Variation at 1 individual SNP site can cause altered binding of the corresponding protein. This altered binding can in turn affect the binding of a 2nd protein at another SNP site, but the extent to which the binding of this 2nd protein is affected will also depend on the variation at the 2nd SNP site.

Associations between LCR/promoter SNPs with height and IGF-I levels are likely to be mediated by GH levels in serum, which may vary due to altered expression of *GHI*. Although the variation in IGF-I SDS between the different genotypes was largely in accordance with the variations in peak GH levels obtained during GH stimulation tests, the association between the genotypes and peak GH levels was not significant (table 3). The fact that IGF-I levels are associated with LCR/promoter SNPs, whereas stimulated GH levels are not, may be explained by the fact that forced GH secretion during a stimulation test does not always reliably reflect the spontaneous GH secretion pattern. Due to the fact that it reflects spontaneous daily GH secretion, serum IGF-I is considered a more reliable indicator of spontaneous GH secretion than GH peaks during stimulation tests [35–37]. Therefore, although LCR/promoter SNPs are not significantly related to peak GH levels obtained during stimulation tests, the relation of these SNPs with height and IGF-I levels is probably mediated by altered spontaneous GH secretion.

The LCR/promoter SNP genotype frequencies and clinical associations differed between IGHD patients and healthy controls. These differences may be explained by the fact that certain genotypes could be beneficial for processes that take place in IGHD patients, but not in controls, due to the altered GH/IGF-I status.

Although all controls had a height within the normal range, the lowest height SDS were found in patients heterozygous for promoter SNPs 6 and 11. This association with shorter stature was specific for the heterozygous state, since controls homozygous for the minor allele had a higher HSDS. The finding that the heterozygous state is associated with a certain phenotype and differs from the 2 homozygous states, has been previously reported and is the subject of research and discussion [9, 38–47]. Heterozygous advantage or disadvantage of promoter SNPs may be explained by allele-dependent regulation of gene expression. A key process in allele-dependent regulation of gene expression is trans-regulation, which means

that an enhancer from 1 allele acts in trans to activate transcription from the promoter of the 2nd allele [45, 46, 48]. Transcription factors can bind 2 DNA molecules simultaneously, functioning as a protein bridge and mediating enhancer-promoter communication between 2 homologues [49]. Trans-regulation, i.e. the interaction of 2 alleles in trans, might explain advantages or disadvantages of heterozygosity as found in several studies [50, 51] and in SNPs 6 and 11 in our study.

Recently, a map of *GHI* promoter SNPs in a Spanish adult control population with normal height was reported [10]. The investigators found 11 SNPs in the promoter region, of which 2 (corresponding to SNPs 8 and 14 in our study) were related to HSDS. The studied promoter SNPs accounted for 6.2% of adult height determination in the adult Spanish population. Horan et al. [9] studied *GHI* LCR and promoter SNPs in relation to height, and found that 3.3% of the variance of adult height was explained by promoter haplotypes. Our study largely analyzed the same set of promoter SNPs as studied by Horan et al. [9] and Esteban et al. [10], but instead of including all promoter SNPs in the combined genotype analysis, our study focused on the SNPs 6, 8, 9 and 11, as they were individually associated with height or IGF-I levels. Our study analyzed combined SNP 6-8-9-11 genotypes with frequencies above 5%. These most frequent combined genotypes were present in 73% of the patients and 68% of the controls. Although genotype numbers were small and results should be interpreted with caution, SNP 6-8-9-11 genotypes explained 10.8% of IGF-I SDS in the IGHD patients ($p = 0.02$) and 15.9% of HSDS in controls ($p = 0.003$). Such a strong association between promoter SNPs with height and serum IGF-I levels has not previously been described, and this may be due to the different approach and the

number of SNPs included in the combined genotype analysis. By limiting the number of SNPs included in the combined genotypes to those that were individually associated with height or IGF-I, we have narrowed the window of promoter SNPs that seem clinically relevant for GH expression, height and IGF-I.

In conclusion, homozygosity for the minor allele of SNP 9 was significantly more frequent among patients than among controls. Since this minor allele at SNP 9 was associated with lower IGF-I levels in IGHD patients, our data suggests that this allele might contribute to impaired GH production in IGHD patients. Apart from SNP 9, SNP 6, 8 and 11 were also related with height and IGF-I levels.

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References

- 1 Goldenberg N, Barkan A: Factors regulating growth hormone secretion in humans. *Endocrinol Metab Clin North Am* 2007;36:37-55.
- 2 Fofanova OV, Evgrafov OV, Polyakov AV, Poltarau AB, Peterkova VA, Dedov II: A novel IVS2 -2A>T splicing mutation in the GH-1 gene in familial isolated growth hormone deficiency type II in the spectrum of other splicing mutations in the Russian population. *J Clin Endocrinol Metab* 2003;88:820-826.
- 3 Miyata I, Eto Y, Kamijo T, Ogawa M, Futakul A, Phillips JA III: Screening for mutations in the GH-1 gene by dideoxy fingerprinting (ddF). *Endocr J* 1999;46(suppl): S71-S74.
- 4 Ogawa M, Kamijo T, Igarashi Y, Nishi Y, Iwatani N, Kohno H, Koga J, Byun YJ: Prevalence of GH-1 gene deletion in patients with isolated growth hormone deficiency in Japan. *GH Gene Study Group. Endocr J* 2000; 47:157-162.
- 5 Osorio MG, Marui S, Jorge AA, Latronico AC, Lo LS, Leite CC, Estefan V, Mendonca BB, Arnhold IJ: Pituitary magnetic resonance imaging and function in patients with growth hormone deficiency with and without mutations in GHRH-R, GH-1, or PROP-1 genes. *J Clin Endocrinol Metab* 2002;87: 5076-5084.
- 6 Salvatori R, Fan X, Phillips JA III, Espigares-Martin R, Martin DL I, Freeman KL, Plotnick L, Al Ashwal A, Levine MA: Three new mutations in the gene for the growth hormone (gh)-releasing hormone receptor in familial isolated gh deficiency type II. *J Clin Endocrinol Metab* 2001;86:273-279.
- 7 Wagner JK, Eble A, Hindmarsh PC, Mullis PE: Prevalence of human GH-1 gene alterations in patients with isolated growth hormone deficiency. *Pediatr Res* 1998;43:105-110.
- 8 Jones BK, Monks BR, Liebhaber SA, Cooke NE: The human growth hormone gene is regulated by a multicomponent locus control region. *Mol Cell Biol* 1995;15:7010-7021.

- 9 Horan M, Millar DS, Hedderich J, Lewis G, Newsway V, Mo N, Fryklund L, Procter AM, Krawczak M, Cooper DN: Human growth hormone 1 (GH1) gene expression: complex haplotype-dependent influence of polymorphic variation in the proximal promoter and locus control region. *Hum Mutat* 2003;21:408–423.
- 10 Esteban C, Audi L, Carrascosa A, Fernandez-Cancio M, Perez-Arroyo A, Ulied A, Andaluz P, Arjona R, Albisu M, Clemente M, Gussinye M, Yeste D: Human growth hormone (GH1) gene polymorphism map in a normal-statured adult population. *Clin Endocrinol (Oxf)* 2007;66:258–268.
- 11 Giordano M, Godi M, Giacomelli F, Lessi M, Mellone S, Paracchini R, Petri A, Bellone J, Ravazzolo R, Bona G, Momigliano-Richiardi P: A variation in a Pit-1 site in the growth hormone gene (GH1) promoter induces a differential transcriptional activity. *Mol Cell Endocrinol* 2006;249:51–57.
- 12 Hasegawa Y, Fujii K, Yamada M, Igarashi Y, Tachibana K, Tanaka T, Onigata K, Nishi Y, Kato S, Hasegawa T: Identification of novel human GH-1 gene polymorphisms that are associated with growth hormone secretion and height. *J Clin Endocrinol Metab* 2000;85:1290–1295.
- 13 Le Marchand L, Donlon T, Seifried A, Kaaks R, Rinaldi S, Wilkens LR: Association of a common polymorphism in the human GH1 gene with colorectal neoplasia. *J Natl Cancer Inst* 2002;94:454–460.
- 14 Wagner JK, Eble A, Cogan JD, Prince MA, Phillips JA III, Mullis PE: Allelic variations in the human growth hormone-1 gene promoter of growth hormone-deficient patients and normal controls. *Eur J Endocrinol* 1997;137:474–481.
- 15 Chanock S: Candidate genes and single nucleotide polymorphisms (SNPs) in the study of human disease. *Dis Markers* 2001;17:89–98.
- 16 Kao SL, Chong SS, Lee CG: The role of single nucleotide polymorphisms (SNPs) in understanding complex disorders and pharmacogenomics. *Ann Acad Med Singapore* 2000;29:376–382.
- 17 Suh Y, Vijg J: SNP discovery in associating genetic variation with human disease phenotypes. *Mutat Res* 2005;573:41–53.
- 18 Wang X, Chen D, Niu T, Wang Z, Wang L, Ryan L, Smith T, Christiani DC, Zuckerman B, Xu X: Genetic susceptibility to benzene and shortened gestation: evidence of gene-environment interaction. *Am J Epidemiol* 2000;152:693–700.
- 19 Baralle M, Baralle FE: Genetics and molecular biology: single nucleotide polymorphism associations and their functional significance. *Curr Opin Lipidol* 2006;17:360–362.
- 20 Cartegni L, Chew SL, Krainer AR: Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet* 2002;3:285–298.
- 21 Mendell JT, Sharifi NA, Meyers JL, Martinez-Murillo F, Dietz HC: Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise. *Nat Genet* 2004;36:1073–1078.
- 22 Liao G, Wang J, Guo J, Allard J, Cheng J, Ng A, Shafer S, Puech A, McPherson JD, Foerzler D, Peltz G, Usuka J: In silico genetics: identification of a functional element regulating H2-Ealpha gene expression. *Science* 2004;306:690–695.
- 23 Adkins RM, Campese C, Vaidya R, Boyd TK: Association between fetal growth restriction and polymorphisms at sites -1 and +3 of pituitary growth hormone: a case-control study. *BMC Pregnancy Childbirth* 2005;5:2.
- 24 Dennison EM, Syddall HE, Rodriguez S, Vropanov A, Day IN, Cooper C: Polymorphism in the growth hormone gene, weight in infancy, and adult bone mass. *J Clin Endocrinol Metab* 2004;89:4898–4903.
- 25 Canzian F, McKay JD, Cleveland RJ, Dossus L, Biessy C, Boillot C, Rinaldi S, Llewellyn M, Chajes V, Clavel-Chapelon F, Tehard B, Chang-Claude J, Linseisen J, Lahmann PH, Pischon T, Trichopoulos D, Trichopoulou A, Zilis D, Palli D, Tumino R, Vineis P, Berrino F, Bueno-de-Mesquita HB, van Gils CH, Peeters PH, Pera G, Barricarte A, Chirlaque MD, Quiros JR, Larranaga N, Martinez-Garcia C, Allen NE, Key TJ, Bingham SA, Khaw KT, Slimani N, Norat N, Riboli E, Kaaks R: Genetic variation in the growth hormone synthesis pathway in relation to circulating insulin-like growth factor-I, insulin-like growth factor binding protein-3, and breast cancer risk: results from the European prospective investigation into cancer and nutrition study. *Cancer Epidemiol Biomarkers Prev* 2005;14:2316–2325.
- 26 Mulhall C, Hegele RA, Cao H, Tritchler D, Yaffe M, Boyd NF: Pituitary growth hormone and growth hormone-releasing hormone receptor genes and associations with mammographic measures and serum growth hormone. *Cancer Epidemiol Biomarkers Prev* 2005;14:2648–2654.
- 27 Ren Z, Cai Q, Shu XO, Cai H, Cheng JR, Wen WQ, Gao YT, Zheng W: Genetic polymorphisms in the human growth hormone-1 gene (GH1) and the risk of breast carcinoma. *Cancer* 2004;101:251–257.
- 28 Wagner K, Hemminki K, Israelsson E, Grzybowska E, Klaes R, Chen B, Butkiewicz D, Pamula J, Pekala W, Forsti A: Association of polymorphisms and haplotypes in the human growth hormone 1 (GH1) gene with breast cancer. *Endocr Relat Cancer* 2005;12:917–928.
- 29 de Graaff LC, Argente J, Veenma DC, Herrebout MA, Friesema EC, Uitterlinden AG, Drent ML, Campos-Barros A, Hokken-Koelega AC: Genetic screening of a Dutch population with isolated growth hormone deficiency. *Clin Endocrinol (Oxf)* 2009;70:742–750.
- 30 Rikken B, van Doorn J, Ringeling A, Van den Brande JL, Massa G, Wit JM: Plasma levels of insulin-like growth factor (IGF)-I, IGF-II and IGF-binding protein-3 in the evaluation of childhood growth hormone deficiency. *Horm Res* 1998;50:166–176.
- 31 Jin Y, Surabhi RM, Fresnoza A, Lytras A, Cattini PA: A role for A/T-rich sequences and Pit-1/GHF-1 in a distal enhancer located in the human growth hormone locus control region with preferential pituitary activity in culture and transgenic mice. *Mol Endocrinol* 1999;13:1249–1266.
- 32 Barrett JC, Fry B, Maller J, Daly MJ: Haplotype: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;21:263–265.
- 33 Courtois SJ, Lafontaine DA, Lemaigre FP, Durviaux SM, Rousseau GG: Nuclear factor-1 and activator protein-2 bind in a mutually exclusive way to overlapping promoter sequences and trans-activate the human growth hormone gene. *Nucleic Acids Res* 1990;18:57–64.
- 34 Giordano M, Godi M, Mellone S, Petri A, Vivenza D, Tiradani L, Carlomagno Y, Ferrante D, Arrigo T, Corneli G, Bellone S, Giacomelli F, Santoro C, Bona G, Momigliano-Richiardi P: A functional common polymorphism in the vitamin D-responsive element of the GH1 promoter contributes to isolated growth hormone deficiency. *J Clin Endocrinol Metab* 2008;93:1005–1012.
- 35 Gandrud LM, Wilson DM: Is growth hormone stimulation testing in children still appropriate? *Growth Horm IGF Res* 2004;14:185–194.
- 36 Hilczner M, Smyczynska J, Stawerska R, Lewinski A: Stability of IGF-I concentration despite divergent results of repeated GH stimulating tests indicates poor reproducibility of test results. *Endocr Regul* 2006;40:37–45.
- 37 Seminara S, Filpo A, Piccinini P, La CF, Cappa M, Faedda A, Loche S: Growth hormone (GH) response to GH-releasing hormone in short children: lack of correlation with endogenous nocturnal GH secretion. *J Endocrinol Invest* 1997;20:118–121.
- 38 Beckman L, Bronnestam R, Cedergren B, Liden S: HL-A antigens, blood groups, serum groups and red cell enzyme types in psoriasis. *Hum Hered* 1974;24:496–506.
- 39 Comings DE, Gonzalez N, Wu S, Gade R, Muhleman D, Saucier G, Johnson P, Verde R, Rosenthal RJ, Lesieur HR, Ruge LJ, Miller WB, MacMurray JP: Studies of the 48 bp repeat polymorphism of the DRD4 gene in impulsive, compulsive, addictive behaviors: Tourette syndrome, ADHD, pathological gambling, and substance abuse. *Am J Med Genet* 1999;88:358–368.
- 40 Comings DE: Molecular heterosis as the explanation for the controversy about the effect of the DRD2 gene on dopamine D2 receptor density. *Mol Psychiatry* 1999;4:213–215.

- 41 Comings DE, MacMurray JP, Gonzalez N, Ferry L, Peters WR: Association of the serotonin transporter gene with serum cholesterol levels and heart disease. *Mol Genet Metab* 1999;67:248–253.
- 42 Frohlander N, Stjernberg N: Association between haptoglobin groups and hereditary predisposition for bronchial asthma. *Hum Hered* 1989;39:7–11.
- 43 Keeney S, Kleckner N: Communication between homologous chromosomes: genetic alterations at a nuclease-hypersensitive site can alter mitotic chromatin structure at that site both in cis and in trans. *Genes Cells* 1996;1:475–489.
- 44 Muller HP, Schaffner W: Transcriptional enhancers can act in trans. *Trends Genet* 1990;6:300–304.
- 45 Ronshaugen M, Levine M: Visualization of trans-homolog enhancer-promoter interactions at the Abd-B Hox locus in the *Drosophila* embryo. *Dev Cell* 2004;7:925–932.
- 46 D’Aiuto L, De Marco R, Edward N, Rizzo A, Chaillet JR, Montecalvo A, Lotze MT, Gambotto A: Evidence of the capability of the CMV enhancer to activate in trans gene expression in mammalian cells. *DNA Cell Biol* 2006;25:171–180.
- 47 Duvillie B, Bucchini D, Tang T, Jami J, Paldi A: Imprinting at the mouse *Ins2* locus: evidence for cis- and trans-allelic interactions. *Genomics* 1998;47:52–57.
- 48 Sipos L, Gyurkovics H: Long-distance interactions between enhancers and promoters. *FEBS J* 2005;272:3253–3259.
- 49 Mahmoudi T, Katsani KR, Verrijzer CP: GAGA can mediate enhancer function in trans by linking two separate DNA molecules. *EMBO J* 2002;21:1775–1781.
- 50 Bozzini C, Girelli D, Bernardi F, Ferraresi P, Olivieri O, Pinotti M, Martinelli N, Manzato F, Friso S, Villa G, Pizzolo F, Beltrame F, Corrocher R: Influence of polymorphisms in the factor VII gene promoter on activated factor VII levels and on the risk of myocardial infarction in advanced coronary atherosclerosis. *Thromb Haemost* 2004;92:541–549.
- 51 Fiedorowicz JG, Moser DJ, Hynes SM, Beglinger LJ, Schultz SK, Ellingrod VL: LA allelic heterozygosity of the 5HTTLPR polymorphism is associated with higher cognitive function and lower interpersonal sensitivity. *Psychiatr Genet* 2007;17:3–4.