Functional aspects of genetic variability in the GH genomic region

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Abstract

Because of the small differences among genotypes, it would be difficult in basal conditions to detect the effect of genetic polymorphism in endocrine function, but this could emerge during provocative tests. We have studied four polymorphic sites of the GH gene region (17q24.2), MSPIA, MSPIB, BGLIIA, and BGLIIB. Gene and haplotype distributions in classes of growth retardation have been studied. The outcome of GH diagnostic test in relation to GH region genotypes has been evaluated by the analysis of area under the GH secretory curve. Ninety-eight growth retarded children have been studied. On the basis of provocative GH test these children were classified as total GH deficit (TD), partial GH deficit (PD), and familial short stature (FSS) with no deficit of GH. Sixty-three healthy controls were also considered. An

increased frequency of MSPIA*2 allele in PD and TD as compared with FSS children and controls has been observed suggesting that this allele is associated with a decreased GH release. BGLIIA*2 allele appears decreased in PD and TD as compared with FSS and controls, suggesting that this allele is associated with an increased release of GH. Carriers of MSPIA*2 allele show a lower GH release as compared with MSPIA*1/*1 subjects on the provocative test by insulin, while carriers of BGLIIA*2 allele show a higher GH release as compared with BGLIIA*1/*1 subjects on the provocative test by clonidine. The functional aspects of genetic variability within the GH genomic area parallel the genetic differences observed between TD and PD versus FSS and control children.

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Introduction

It is likely that 'normal' genetic variability of enzymes and other cellular structures influences endocrine function resulting in different susceptibility to diseases and/or their clinical expressions. However, because of the small differences among genotypes, it would be difficult to detect the effect of such variability in basal conditions. On the contrary, measurable differences may emerge following provocative tests.

Polymorphic sites in the growth hormone (GH) gene region have been mainly studied in relation to evolutionary problems of the GH cluster (Chakravarti *et al.* 1984) rather than to their possible importance as markers of functional variability of transcribed genes and therefore to their possible clinical relevance. In the present note, we have studied four polymorphic sites of the GH gene region (17q24.2). Gene and haplotype distributions in classes of growth retardation have been analyzed and compared with controls. The outcome of a GH diagnostic test in relation to GH region genotypes has been evaluated by the analysis of area under the GH secretory curve. We have also considered possible interaction between GH region genotypes and acid phosphatase (ACP1; Bottini *et al.* 2001, 2002a) in relation to GH release during provocative tests.

GH genomic area and polymorphic sites studied

The human GH protein is codified by *GH1* gene that is the first of a cluster of five genes located on the long arm of chromosome 17 (q24.2) within a 50 kb cluster of five related genes (Fig. 1). The other four genes have a >90% sequence identity with the *GH1* gene. The other genes are *CSH1* and *CSH2* that encode the same human chorionic somatomammotropin (CS protein), placental growth hormone gene (*GH2*), and a partially disabled pseudo gene (*CSHL1*).

In this genomic area, we have considered four polymorphic sites: MSPIA, MSPIB, BGLIIA, and BGLIIB (Chakravarti *et al.* 1984; Fig. 2).

Materials and Methods

Ninety-eight growth retarded children in the population of Ancona, Italy have been studied. Insulin and clonidine stimulation tests were performed in all subjects. The GH value (plasma level in micrograms per liter) at a fixed point of the test was considered for classification into three categories: total deficit (TD), partial deficit (PD), and familial short stature (FSS). For insulin stimulation, the cut-off points were 4 (PD versus TD) and 8 (FSS versus

Figure 1 Map of the gene region examined.

PD) $\mu\text{g/l}$ for the level of GH at 60 min from the beginning of the test, and for clonidine testing 6 and 10 $\mu\text{g/l}$ respectively at 120 min from the beginning of the test were used. For clinical purposes, the level at a fixed point has some practical advantages over the peak level since the latter would require a higher number of determinations of GH level during the provocative test. Both test results had to be below the cut-off point in order to include the patient in the lower class. Based on the results of GH stimulation tests with insulin and clonidine among children in whom genotypes of GH area were determined, 23 were classified as FSS, 52 as PD, and 23 as TD of GH. With the exception of two subjects (one TD and one PD), who showed an associated deficit of thyrotropin, all cases had isolated GH deficiency. No other evident cause of growth retardation such as malabsorption was present, and no sign of sellar area lesion was detectable.

Mean age was 11.3 years for FSS, 10.3 years for PD, and 9.9 years for TD. Deviation from mean stature was -2.11 s.d.,

-2.08 s.d., and -2.00 s.d. respectively. The sex ratio was 2.00 for FSS, 2.41 for PD, and 1.55 for TD.

Informed consent was obtained from the parents of children prior to testing. The investigation was performed a few years ago in collaboration with the Laboratory of Genetics of the University of Camerino and the Institute of Pediatric Clinic of the University of Ancona. At present, it is noteworthy that, insulin challenge testing is no longer used in Italian pediatric clinics.

Sixty-three healthy adults (35 males and 28 females) from the same population were studied as controls. It was not possible to study healthy children as controls.

It was not possible to determine the genotype of the four sites studied in all subjects, thus the number of subjects shown in the tables differs among sites. The subsets studied were random samples of the whole sample.

Some data on these subjects have been included in a preliminary note concerning the relationship between GH genomic region and glycemic level in type 2 diabetes (Bottini *et al.* 2002b).

RFLPs											
	Sizes (bp)	Alleles	BGI II-A	Alleles	BGI II-B	Alleles	MSP I-A	Alleles	MSP I-B	Sizes (bp)	
17-600	—									23-130	
13-800	—	2 (–)	—								
8-100	—	1 (+)	—	2 (–)	—					9-420	
6-000	—									6-580	
						2 (–)	—			4-360	
						1 (+)	—	2 (–)	—		
2-970	—			1 (+)	—			1 (+)	—		
879	—									2-320	
										2-000	

Figure 2 Sizes of alleles at the four polymorphic sites examined. For each polymorphism the shorter fragment is denoted by 1 and the longer fragment by 2 that correspond to the presence (+) or absence (–) of a restriction site at a particular DNA location.

DNA analysis

Genomic DNA extracted from peripheral blood samples was processed by conventional Southern blot analysis (Sambrook *et al.* 1989). From each total genomic DNA, 8 µg were digested overnight according to the conditions specified by the supplier (Promega). Following electrophoresis in 0.7–1.2% (wt/vol) agarose gel in Tris–acetate/EDTA buffer, DNA was blotted overnight onto Hybond-N nylon membrane (Amersham) and fixed by baking at 90 °C. The filters were prehybridized (HB-ID) at 65 °C for 18 h in 6×SSC 0.5% in SDS and 0.5% Denhardt solution plus 3×10⁷ c.p.m. probe that was radiolabeled to a specific radioactivity >10¹⁰ c.p.m./µg DNA by a nick-translation system (Promega) using [α -³²P]dATP (Amersham). All filters were washed twice for 30 min at 65 °C in 2×SSC plus 0.1% SDS and once for 30 min at 65 °C in 0.1×SSC plus 0.1% SDS. The filters were exposed to X-ray films using an intensifying screen at –70 °C.

RFLP, probes, and restriction enzymes

The four polymorphisms have been examined using the probe C-H800 (Chakravarti *et al.* 1984) and the restriction enzymes MSPI and BGLII. The size of alleles is shown in Fig. 2.

Analysis of area under GH secretory curve

The area under the GH secretory curve depicting the rise of plasma GH as a function of time has been considered as a measure of total GH released into the plasma during the provocative test (Koppeschaar *et al.* 2004). This quantity has been correlated with the genotype of each polymorphic site of GH area.

Interaction with ACP1

We have reconsidered a previous study (Bottini *et al.* 2001) showing an effect of ACP1 genetic variability on the area under the GH secretory curve during provocative tests.

Statistical analysis

Statistical analyses were performed by SPSS programs (SPSS/PC + version 5.0, 1995; SPSS Inc., Chicago, IL, USA).

Haplotype frequencies are maximum likelihood estimates (program MENDEL, Department of Biostatistics, University of Michigan, Ann Harbor, MI, USA).

Results

Alleles and haplotype analysis

Table 1 shows the proportion of alleles at the four polymorphic loci in children with growth retardation and in controls.

Table 1 Alleles of growth hormone (GH) area

	Alleles		
	MSPIA*1 (%)	MSPIA*2 (%)	No. of alleles
FSS	55	45	40
PD	52	48	102
TD	50	50	46
Controls	66	34	82
Significance of difference			
	χ^2	P	
FSS versus controls	0.926	0.336	
PD versus TD	0.0018	0.965	
PD + TD versus controls	3.945	0.047 (0.188)	
PD + TD versus FSS	0.0535	0.816	
	Alleles		
	MSPIB*1 (%)	MSPIB*2 (%)	No. of alleles
FSS	63	37	38
PD	65	35	102
TD	61	39	44
Controls	78	22	68
Significance of difference			
	χ^2	P	
FSS versus controls	1.988	0.158	
PD versus TD	0.0391	0.843	
PD + TD versus controls	3.709	0.0541	
PD + TD versus FSS	0.0163	0.898	
	Alleles		
	BGLIIA *1 (%)	BGLIIA *2 (%)	No. of alleles
FSS	56	43	46
PD	80	20	100
TD	80	20	46
Controls	60	40	126
Significance of difference			
	χ^2	P	
FSS versus controls	0.0746	0.784	
PD versus TD	0.263	0.871	
PD + TD versus controls	11.947	0.000 (0.004)	
PD + TD versus FSS	9.057	0.003 (0.01)	
	Alleles		
	BGLIIB *1 (%)	BGLIIB *2 (%)	No. of alleles
FSS	59	41	46
PD	51	49	102
TD	45	54	44
Controls	65	35	120
Significance of difference			
	χ^2	P	
FSS versus controls	0.329	0.566	
PD versus TD	0.187	0.665	
PD + TD versus controls	5.968	0.015 (0.060)	
PD + TD versus FSS	0.885	0.347	

Significance (P) after correction for multiple comparisons are shown in parenthesis.

Table 2 Pairwise haplotypes of GH area

Haplotype					No. of haplotypes
	*1/*1 (%)	*1/*2 (%)	*2/*1 (%)	*2/*2 (%)	
MSPIA–MSPIB					
FSS	53	0	10	37	38
PD	47	5	18	30	102
TD	43	7	2	32	44
Controls	68	0	10	22	68
		Significance of difference			
		χ^2	<i>P</i>		
FSS versus controls		4.391	0.111		
PD versus TD		0.814	0.846		
PD + TD versus controls		8.551	0.036 (0.144)		
PD + TD versus FSS		1.605	0.658		
Haplotype					No. of haplotypes
	*1/*1 (%)	*1/*2 (%)	*2/*1 (%)	*2/*2 (%)	
MSPIA–BGLIIA					
FSS	18	37	37	8	38
PD	34	21	41	3	94
TD	40	12	43	5	42
Controls	32	33	38	2	78
		Significance of difference			
		χ^2	<i>P</i>		
FSS versus controls		3.670	0.299		
PD versus TD		1.928	0.587		
PD + TD versus controls		6.320	0.097		
PD + TD versus FSS		8.713	0.033 (0.132)		
Haplotypes					No. of haplotypes
	*1/*1 (%)	*1/*2 (%)	*2/*1 (%)	*2/*2 (%)	
MSPIA–BGLIIB					
FSS	50	5	8	37	40
PD	37	20	14	29	92
TD	35	18	10	38	40
Controls	59	6	5	29	78
		Significance of difference			
		χ^2	<i>P</i>		
FSS versus controls		1.247	0.741		
PD versus TD		1.040	0.790		
PD + TD versus controls		14.018	0.003 (0.012)		
PD + TD versus FSS		6.306	0.098		
Haplotypes					No. of haplotypes
	*1/*1 (%)	*1/*2 (%)	*2/*1 (%)	*2/*2 (%)	
MSPIB–BGLIIA					
FSS	26	37	29	8	38
PD	42	22	33	2	94
TD	45	17	38	0	42
Controls	44	3	19	4	66

	Significance of difference	
	χ^2	<i>P</i>
FSS versus controls	3.816	0.282
PD versus TD	1.616	0.655
PD + TD versus controls	8.697	0.036 (0.144)
PD + TD versus FSS	9.978	0.019 (0.076)

Haplotypes					No. of haplotypes
MSPIB-BGLIIA	*1/*1 (%)	*1/*2 (%)	*2/*1 (%)	*2/*2 (%)	
FSS	53	10	5	32	38
PD	42	23	8	27	92
TD	40	23	5	33	40
Controls	62	15	2	21	66

	Significance of difference	
	χ^2	<i>P</i>
FSS versus controls	2.964	0.397
PD versus TD	0.592	0.898
PD + TD versus controls	8.458	0.037 (0.148)
PD + TD versus FSS	3.188	0.363

Haplotypes					No. of haplotypes
BGLIIA-BGLIIB	*1/*1 (%)	*1/*2 (%)	*2/*1 (%)	*2/*2 (%)	
FSS	16	41	43	0	44
PD	28	48	22	0	102
TD	28	51	16	5	43
Controls	26	35	39	0	120

	Significance of difference	
	χ^2	<i>P</i>
FSS versus controls	1.805	0.405
PD versus TD	2.608	0.456
PD + TD versus controls	13.345	0.004 (0.016)
PD + TD versus FSS	14.288	0.003 (0.012)

Significance (*P*) after correction for multiple comparisons are shown in parenthesis.

For all loci no significant difference is observed between PD and TD. With the exception of MSPIB, the difference between controls and PD plus TD is always statistically significant. No significant difference is observed between FSS and controls. A statistically significant difference between FSS and PD plus TD ($P=0.003$; $P=0.01$ after correction for multiple comparisons) is observed for BGLIIA. In particular, BGLIIA*1 allele is represented much more in PD and TD than in FSS and controls.

No significant difference has been observed between males and females concerning allele frequency either in controls or in growth retarded children. In growth retarded children, no significant effect of age on allele frequency has been observed.

Table 2 shows the distribution of haplotypes for the six pairs of loci. No significant difference is observed between PD and TD.

In five haplotypic classes a significant difference has been observed between PD plus TD and controls. The difference is particularly marked for BGLIIA-BGLIIB and MSPIA-BGLIIB haplotypes. No significant difference is observed between FSS children and controls. In three haplotypic classes significant differences are observed between PD plus TD and FSS. Such differences are particularly marked for the BGLIIA-BGLIIB and MSPIB-BGLIIA.

Area under the GH secretory curve

Table 3 shows the plasma GH released during 60 min after insulin stimulation in relation to genotype of polymorphic sites studied. Owing to the very low GH released, TD children have not been considered in this analysis. Moreover, since there are few *2/*2 homozygotes, we have joined *1/*2 heterozygotes

Table 3 Plasma GH released (area under secretory curve) during 60' after insulin stimulation in relation to MSPIA, MSPIB, BGLIIA, and BGLIIB genotypes in subjects with familiar short stature (FSS) and subjects with partial deficit (PD) of GH

	Genotype				Significance of difference between genotypes (<i>P</i>)
	*1/*1		*1/*2 + *2/*2		
	Mean	S.E.M	Mean	S.E.M	
MSPIA					
FSS	10.78	2.57	6.60	0.72	0.03
PD	3.68	0.41	2.70	0.33	0.10
Cumulative probability (<i>P</i>)					0.023
MSPIB					
FSS	9.63	2.15	6.60	0.74	0.09
PD	3.26	0.39	2.84	0.34	0.47
Cumulative probability (<i>P</i>)					0.170
BGLIIA					
FSS	7.53	1.65	7.12	0.81	0.81
PD	3.05	0.34	3.17	0.34	0.80
Cumulative probability (<i>P</i>)					0.920
BGLIIB					
FSS	7.47	1.57	6.78	0.79	0.66
PD	2.74	0.57	3.31	0.27	0.33
Cumulative probability (<i>P</i>)					0.470

with *2/*2 homozygotes (carriers of *2 allele). MSPIA influences GH release, the presence of *2 allele is associated with lower level of GH plasma release. No appreciable difference concerning GH release during stimulation with insulin is observed in relation to BGLIIA and BGLIIB genotypes.

Figure 3 shows plasma GH concentration during stimulation with insulin in FSS children in relation to MSPIA genotype. Carriers of *2 allele reach a maximum GH level at 30 min while *1/*1 children reach the maximum at 45 min. Plasma GH level during the test is always higher in *1/*1 genotypes than in carriers of *2 allele.

Table 4 shows the plasma GH released during 120 min after stimulation with clonidine in relation to the genotype of polymorphic sites considered. No appreciable difference concerning GH release is observed in relation to MSPIA and MSPIB genotypes. BGLIIA, on the contrary, influences GH release; the presence of *2 allele is associated with higher levels of GH plasma release.

Figure 4 shows GH plasma concentration during stimulation with clonidine in FSS children in relation to BGLIIA genotype. *1/*1 children reach a maximum GH level at 60 min while carriers of *2 allele does not yet reach the maximum at 120 min. After 60 min, plasma GH level is always much higher in carriers of *2 allele than in *1/*1 children.

The pattern of these differences in relation to genotype is always much more evident in FSS than in PD children.

In a previous note, we have shown that the area under the GH secretory curve is greater in subjects with medium-low ACP1 activity than in those with high activity (Bottini *et al.* 2001). ACP1 is a protein tyrosine phosphatase that is able to dephosphorylate the insulin receptor, thus decreasing the effects of insulin (Bottini *et al.* 2002a). This suggests a possible positive interaction between low ACP1 activity genotypes

(A and BA) and MSPIA*1/*1 genotype. In fact, low activity ACP1 genotypes (A and BA) enhance the effect of MSPIA*1/*1 genotype during stimulation by insulin ($P=0.12$ in FSS and $P=0.015$ in PD, data not shown). On the other hand, low activity ACP1 genotypes (A and BA) do not enhance the effect of BGLIIA*1/*2 and *2/*2 genotypes during stimulation by clonidine, giving support to the hypothesis of different mechanisms of action of the two GH genomic areas on GH release.

Discussion

An increased frequency of MSPIA*2 allele in partial and total GH deficit when compared with FSS children and controls has been observed suggesting that this allele is associated with

Figure 3 Subjects with short familiar stature. GH plasma concentration after stimulation by insulin according to MSPIA genotypes.

Table 4 Plasma GH released (area under secretory curve) during 120 min after clonidine stimulation in relation to MSPIA, MSPIB, BGLIIA, and BGLIIB genotypes in subjects with FSS and subjects with PD of GH

	Genotype				Significance of difference between genotypes (<i>P</i>)
	*1/*1		*1/*2 + *2/*2		
	Mean	S.E.M	Mean	S.E.M	
MSPIA					
FSS	10.64	3.80	9.10	1.44	0.66
PD	7.65	1.27	8.90	0.84	0.44
Cumulative probability (<i>P</i>)					0.650
MSPIB					
FSS	10.25	2.57	8.87	1.69	0.65
PD	9.96	1.29	7.92	0.83	0.17
Cumulative probability (<i>P</i>)					0.350
BGLIIA					
FSS	4.11	2.67	10.51	1.36	0.02
PD	7.40	0.85	8.71	1.05	0.33
Cumulative probability (<i>P</i>)					0.040
BGLIIB					
FSS	7.53	2.28	9.16	1.53	0.55
PD	9.52	1.09	7.40	0.81	0.17
Cumulative probability (<i>P</i>)					0.320

a decreased GH release as compared with the MSPIA*1 allele. On the other hand, the BGLIIA*2 allele appears strongly decreased in PD and TD as compared with FSS and controls suggesting that this allele is associated with an increased release of GH.

Carriers of the MSPIA*2 allele show a lower GH release as compared with MSPIA *1/*1 subjects on the provocative test by insulin, while carriers of the BGLA*2 allele show a higher GH release as compared with BGLIIA *1/*1 subjects on the provocative test by clonidine. Thus, the functional aspects of genetic variability within the GH genomic area parallel the genetic differences observed between TD and PD versus FSS and controls. This concordance between genetic differences and functional aspects reinforces the inference on the

association between genotype and GH release shown in Tables 3 and 4. Indeed, considering the data in Tables 1 and 2, the *a priori* probability to find such an association is definitively different from zero; moreover the data in Tables 1 and 2 suggest a direction for such association. Therefore, the level of significance for MSPIA insulin (Table 3) and BGLIIA clonidine (Table 4) is higher ($P=0.012$ and 0.020 respectively).

The MSPIA site is associated with outcome of insulin stimulation, while the BGLIIA site is associated with the outcome of clonidine stimulation. This is an important difference that deserves further investigation. Different chemical substances may induce the same clinical relevant effect acting on different metabolic pathways that are regulated by different genetic systems. From our data, it seems that the increased release of GH after stimulation by insulin depends on a genetic site that does not influence the effects of clonidine and *vice versa*.

The great majority of growth retardation cases have a multifactorial origin with a contribution of many genes. The present data suggest that ACP1 interacts with sites in the GH genomic region concerning the effect on GH release. This observation may have clinical relevance to detect the genes involved in the susceptibility to growth retardation and in the release of GH during the provocative tests.

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Figure 4 Subjects with short familial stature. GH plasma concentration after stimulation by clonidine according to BGLIIA genotypes.

of interest that would prejudice the impartiality of this scientific work.

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