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Analysis of the putative regulatory region of the gastric inhibitory polypeptide receptor gene in food-dependent Cushing's syndrome

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Abstract

Gastric inhibitory polypeptide (GIP)-dependent Cushing's syndrome (CS) results from the ectopic expression of non-mutated GIP receptor (hGIPR) in the adrenal cortex. We evaluated whether mutations or polymorphisms in the regulatory region of the GIPR gene could lead to this aberrant expression. We studied 9.0 kb upstream and 1.3 kb downstream of the GIPR gene putative promoter (pProm) by sequencing leukocyte DNA from controls and from adrenal tissues of GIP- and non-GIP-dependent CS patients. The putative proximal promoter region (800 bp) and the first exon and intron of the hGIPR gene were sequenced on adrenal DNA from nine GIP-dependent CS, as well as on leukocyte DNA of nine normal controls. Three variations found in this region were found in all patients and controls; at position −4/−5, an insertion of a T was seen in four out of nine patients and in five out of nine controls. Transient transfection studies conducted in rat GC and mouse Y1 cells showed that the TT allele confers loss of 40% in the promoter activity. The analysis of the 8-kb distal pProm region revealed eight distal single nucleotide polymorphisms (SNPs) without probable association with the disease, since frequencies in patients and controls were very similar. In conclusion, mutations or SNPs in the regulatory region of the GIPR gene are unlikely to underlie GIP-dependent CS.

Keywords: Adrenal Cushing's syndrome; Ectopic receptors; GIP receptor promoter

1. Introduction

Primary adrenal etiologies are responsible for 15–20% of endogenous Cushing's syndrome (CS) in adults and are the most frequent cause in childhood. In most cases, the regulation of cortisol secretion in adrenal CS, where ACTH is suppressed, is poorly understood. No activating mutations in ACTH receptor gene have been identified in adrenal tumors [1,2]. In primary pigmented micronodular adrenal disease (PPNAD), mutations in the gene coding for the regulatory subunit R1A of protein kinase A (PRKAR1A), leading to an increased cAMP-stimulated activity, have been described [3–5]. Mutation of the α subunit of the G protein, which constitutively activates steroidogenesis, is present in adrenocortical cells leading to adrenal nodules, and hypercorticism was described in some cases of McCune–Albright's syndrome [6] and in patients with ACTH-independent macronodular hyperplasia (AIMAH) with no other features of this syndrome [7].

In some unilateral cortisol-producing adrenal adenomas and most cases of ACTH-independent bilateral macronodular adrenal hyperplasia, steroidogenesis can be regulated via the aberrant expression or function of one or various hormone receptors such as those for gastric inhibitory polypeptide (GIP), β-adrenergic agonists, luteinizing hormone/human chorionic gonadotropin, vasopressin (V1-AVPR), and serotonin (5-HT_4R) [8–12].

Food-dependent CS was initially reported in 1987 [13], and in vivo and in vitro studies demonstrated the abnormal regulation of cortisol secretion by GIP in this clinical condition [14–16]. GIP-dependent CS was found to result from the ectopic expression of non-mutated GIPR in adrenal tissues [16–21]. So far, at least 24 cases of GIP-dependent CS have been reported, in AIMAH and in unilateral adenomas [12–21]. In GIP-dependent adrenal tissues, cortisol-stimulating effects of GIP are coupled to an increase of cAMP, but not of IP_3 production [20,21]. In normal human adrenals, GIP stimulation does not increase cortisol secretion [14,20,21]; a small amount of GIPR

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mRNA occasionally found in human adrenals may reflect its expression in endothelial cells [22,23].

The molecular mechanisms underlying the ectopic expression and function of the membrane hormone receptors are still unknown. Mutations in the coding regions of the GIPR gene were not found in GIP-dependent cases [18,21,24]. The pathophysiology of this syndrome addresses the question of tissue-specific gene expression. In AIMAH, the mutational events must have occurred early in the embryogenesis so that all cortical cells would carry the defect, which is polyclonal; in cases of unilateral adenomas, that have a monoclonal origin, the defect could be a somatic mutation [12]. One possible mechanism could be the presence of mutations or polymorphisms in regulatory regions of the GIP receptor gene altering its expression in adrenal glands by creating or abrogating a binding site for an adrenocortical-specific transcription factor/repressor/co-factor complex.

GIP is a 42-amino acid peptide secreted by K cells of the duodenum and small intestine, in response to food absorption [25], and it acts in concert with glucagon-like peptide (GLP)-1 as the major incretins of glucose-induced insulin secretion [26,27]. GIP exerts its effects by binding to its specific GIP receptor (GIPR). GIPR is expressed in a large number of tissues. In addition to its well-known insulinotropic effect, GIP exerts other physiologic [28–31] and pathophysiologic effects [32–34].

The hGIPR gene is located on chromosome 19q13.2–13.3 and encodes a predicted 466-amino acid protein with 79% identity to the rat homologue [22,35]. Several isoforms have been described; however, only the predicted form with 466 amino acids and another one with a 27-amino acid insertion at the carboxy-terminal region are functional [18,36,37]. The regulatory region of the GIPR gene has been characterized in rats; it is a GC-rich region and contains no TATA box or CAAT motif, but has an initiator element located close to the transcription start site. Transcription binding motifs include a cAMP response element, an octamer-binding site and three SP1 sites; moreover, the region 200 bp upstream seems to repress GIPR transcription in cells that normally do not express this gene [38]. The human GIPR promoter is currently being characterized in our laboratory; its structure and functional elements in the putative proximal promoter are very similar to those of the rat GIPR promoter [39]. In this study, we examined if alterations in the regulatory regions of the GIPR gene underlie the adrenal overexpression of the GIPR gene in GIP-dependent CS.

2. Material and methods

2.1. Patients and controls

DNA was obtained from adrenal tissues of nine patients with GIP-dependent CS (seven AIMAH; two unilateral adenomas); these patients were previously described [24,40,41]. Adrenal DNA from one patient with Cushing's disease, one patient with vasopressin-dependent CS [42], and leukocyte DNA from nine healthy individuals were used as controls. The institutional ethics committee approved the study protocol.

2.2. DNA analysis

2.2.1. In silico analysis of putative promoter (pProm)

The characteristics such as nucleotide profile, repeated sequences distribution, and the identification of potentials regulatory elements-binding sites were realized using software programs such as BLAST (http://ncbi.nlm.nih.gov/BLAST), the Mac Vector DNA analysis program (version 6.5.1, 1999, Oxford Molecular Group plc), software for research of simple repeated sequences, CpG islands (http://repeatmasker.-genome.washington.edu/ and http://www.cbi.ac.uk), and for sites of potentials binding of transcription factors, Tess(http://www.cbil.upenn.edu/tess), Transfac (http://transfac.-gbf.de), and TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html).

2.2.2. DNA preparation

At surgery, adrenal tissues were rapidly collected, frozen in liquid nitrogen, and stored at −80 °C until analysis. DNA was extracted using either the classical method of alkaline shock or Trizol® (Gibco BRL, Life Technologies, Burlington, Canada). Leukocyte DNA was extracted with QIAEX kit (Gibco BRL, Life Technologies, Burlington, Canada).

2.2.3. Polymerase chain reaction (PCR)

The 9-kb upstream region and the 1.3-kb downstream region of the GIPR gene (Cosmid R28204c19—GenBank accession number AC006132) were amplified in several fragments of variable size using the primers listed in the Table 1. PCR reactions contained 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each of deoxy-NTP, 20 pmol each sense and antisense primers, 100–200 ng DNA, and 2.5 U Taq DNA polymerase. Thermal cycling consisted of an initial denaturation at 94 °C for 3 min followed by 30–40 cycles of denaturing at 94 °C (30 s), annealing at the temperature specific for each pair of primers (15–30 s), and extension at 72 °C (60–120 s), with a final 7 min extension at 72 °C. PCR fragments were isolated by electrophoresis in 1–2% agarose gel and extracted using the filter paper DAE81, and purified with phenol–chloroform.

2.2.4. DNA sequencing

The PCR products were directly sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) on an automated DNA-sequencer ABI PRISM 310 (Applied Biosystems/Perkin-Elmer). The primers used in sequencing are shown in Table 1. In some fragments, direct sequencing of PCR products was difficult due to the presence of highly repetitive regions (Ala11A–Ala9AS, Ala17S–Ala15AS, Ala25S–25As); in these cases, PCR products were subcloned using the

Table 1
Oligonucleotides used for PCR amplification and sequencing the putative regulatory region of the hGIPR gene

Oligonucleotide	Sequence (5′–3′)	Position
24s	AGCCTTCCCAACAGACAGG	−8654/−8637
28s	GTTGGGCATGGGCATGGTAGGCAC	−8315/−8298
27s	ACCAGGCATCTACCCACCCCTGAC	−7283/−7260
25s	GCTACCGCACCAGGCCTTTTCTGG	−6508/−6494
36s	TCTCTCCGGCTCAGTCCCTG	−5893/−5874
37s	GCCACTGTGTCTGGCCTCTCTTCTTG	−5396/−5371
38s	ACTGCCTTCCTCTGTGGAGT	−4161/−4140
17s	GAGGGTGACGGTGTGAG	−3337/−3320
14s	AGGGAAGGAGACAAGGGT	−2002/−1985
11s	CCAGCCTGGGCAACATGGTG	−949/−930
10s	GCGAGTAACCACACAACTGCATACTGC	−729/−702
9s	GACTGTGCCTTTCCATGGC	−335/−317
8s	CTCCCCGCTTCCGTTCCCT	−104/−84
23as	CTGGGCATCAGAGTGAGAC	−7221/−7239
22as	TACTTGGGAGGCAGAGGCAG	−6360/−6379
36as	ATGGTGGTGGTGCGCCTGTGG	−5155/−5175
25as	CTCGCTTCCTGGACTTAGGCTGCC	−3958/−3981
26as	AGTCACCCACAGAGTCCTCAGTC	−3252/−3274
15as	ATGGCACCCAACGGGATG	−1960/−1977
10as	GCCATTGGAAAGGCACAGTC	−317/−335
9as	TGTCCCCAGTCCTGCTCCTG	+53/+34
i2as	CATCAAGAGACAGAGATGA	+201/+183
i1as	GCCCAGAGTAAGTGGAACA	+620/+602
2as	CGGCGGCGAAGGGTCTGGTTCGTGCA	+1271/+1246

The first nucleotide of the TSS is considered +1.

Gateway® system and sequenced (InVitrogen-Gibco, Burlington, Canada). For the fragment Ala25S–25AS, an internal fragment was obtained using Ala36S–Ala36AS and subsequently subcloned. When subcloning was necessary, five clones were sequenced in each construction in both sense and antisense directions. Sequences containing nucleotide variations were sequenced at least twice.

2.2.5. Transfection study

2.2.5.1. DNA construct. The −103 to +1271 hGIPR promoter/exon/intron region was amplified from DNA of two GIP-dependent CS adrenals, one presenting a single T at −4 and the other presenting a T/TT at the same position. The amplification was obtained using the sense primer, 5′-AAAAAGCAGGCTCTCCCCGCTTCCGTTCCCT-3′ and the antisense primer, 5′-AGAAAGCTGGGTCGGCGGCGAAGGGTCTGGTTCGTGCA-3′. Cloning in GWpGL3-Basic (luciferase vector) was done with the Gateway™ technology (Invitrogen), according to the manufacturer's instructions; the presence of T or TT at −4 and orientation was confirmed by dideoxy termination sequencing (Pharmacia).

2.2.5.2. Transient transfection. Mouse adrenal Y1 cells and rat lactosomatotroph GC cells were seeded at 0.8×10^6 cells per plate in 60-mm culture dishes with DMEM 10% FBS, 1× penicillin, and 1× streptomycin. After 24 h, transient transfection was made by the calcium phosphate method, using 5.0 μg of the construction and 2.5 μg of RSV β-gal. Forty-eight hours post transfection cells were lysed and assayed for luciferase activity in a Dynatech ML 2250 luminometer; results were normalized with β-galactosidase activity and were calculated as fold increase of the control. Each cell line was assayed twice, independently.

3. Results

3.1. Structural analysis of the putative promoter region of the GIPR gene

Although not completely functionally characterized in humans, the pProm region of the GIPR gene was cloned and sequenced by the Human Genome Project. For our study, we considered a region of 9 kb upstream the transcription start site (TSS) of the GIPR gene, the distal 5′ region flanking a polyA tail of a yet unidentified gene.

3.1.1. GC content and CpG islands

The GC content of the GIPR pProm greatly varies (20–65%), suggesting that different regions have a higher GC content. In the proximal region of pProm (−1 to −200 bp), there is a GC-rich region that harbors several potentials TF-binding sites (see Section 3.1.2). Three other distal regions (−2900/−3200, −3700/−4200, −6040/−6720 bp) are also rich in GC (60–70%). Moreover, four potential CpG islands were revealed by computational analysis, including one in the proximal region of pProm,

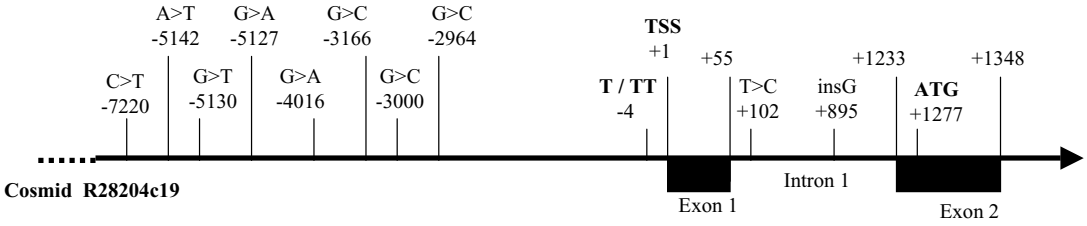

Fig. 1. Position of SNPs found in the putative regulatory region of the GIPR gene in GIP-dependent CS patients and in controls.

but they are short (<200 bp), especially the one near the TSS, which has only 70 bp.

3.1.2. DNA motifs

Analysis of the DNA sequence of the promoter region of GIP-R gene 350 bp upstream to the transcription start site revealed the absence of TATA or CAAT boxes, but the presence of several consensus sites for GC and GT boxes with potential interaction with Sp1 and Sp3 transcription factors. Binding sites for CREB, AP-1, and Oct-1 transcription factors identified in the rat promoter [38] are not observed in the proximal human sequence.

3.2. Single nucleotide polymorphisms (SNPs) in the putative promoter region of the GIPR gene

A total of 11 SNPs were identified within the 9-kb upstream region the GIPR gene TSS and the 1.3-kb downstream region in GIP-dependent adrenal tissues and in normal controls (Fig. 1; Table 2).

3.2.1. SNPs in the distal region of pProm

A total of eight polymorphisms were identified within the distal region (−0.8 to −9.0 kb) of the putative promoter region of the GIPR gene in DNA of GIP-dependent adrenal tissues and in genomic DNA from the controls (Table 2). Change 1 was C → T or C → G substitution at nucleotide −7220. Change 2 was A → T substitution at nucleotide −5142. Change 3 was G → T substitution at nucleotide −5130. Changes 4 and 5 were G → A substitution at nucleotides −5127 and −4016, respectively. Changes 6, 7, and 8 were G → C at nucleotides −3166, −3000, and −2964, respectively. Polymorphisms C-7220T/G, G-4016A, G-3166C, G-3000C, and G-2964C seem to occurs at high frequency (>50%), both in patients and in controls. Polymorphisms G-5130T and G-5127A seem to be less frequent, both in patients (1/4) and in controls (1/9). Polymorphism A-5142T seems to be less frequent in patients (1/3) than in controls (9/9), but only a small number of patients were studied. Polymorphisms 1, 7, and 8 are located within *Alu* sequences. Polymorphisms 2, 3, and 4 are located within type $(A)_{3–16}$ microsatellite region. Polymorphisms 7 and 8 are located in a possible CpG island, but none of them includes a CpG dinucleotide.

3.2.2. SNPs in the proximal region of pProm

In this report, numbering of the putative GIPR promoter is relative to the first nucleotide of exon 1. The DNA sequencing of the proximal pProm region in the nine GIP-dependent adrenal tissues revealed a SNP at position −4/−5 bp; 5/9 patients presented a single T at this position,

Table 2
Distribution of genotypes and allelic frequency for the putative regulatory region in GIPR gene, in GIP-dependent CS and controls

Polymorphism	Genotypes			Alleles	
−7220	CC	CT	TT	C	T
GIP-AIMAH	33.3	0	66.6	33.3	66.6
Control	33.3	22.2	44.4	44.4	55.5
−5142	AA	AT	TT	A	T
GIP-AIMAH	66.6	33.3	0	91.6	8.3
Controls	0	77.7	22.2	38.8	61.1
−5130	GG	GT	TT	G	T
GIP-AIMAH	80.0	20.0	0	95.0	5.0
Controls	88.8	11.1	0	94.4	5.5
−5127	GG	GA	AA	G	A
GIP-AIMAH	80.0	20.0	0	95.0	5.0
Controls	88.8	11.1	0	94.4	5.5
−4016	GG	GA	AA	G	C
GIP-AIMAH	0	16.6	83.3	8.3	91.6
Controls	0	44.4	55.5	22.2	77.7
−3166	GG	GC	CC	G	C
GIP-AIMAH	0	100	0	50	50
Controls	33.3	44.4	22.2	55.5	44.4
−3000	GG	GC	CC	G	C
GIP-AIMAH	0	40.0	60.0	20.0	80.0
Controls	0	11.1	88.8	5.5	94.4
−2964	GG	GC	CC	G	C
GIP-AIMAH	0	33.3	66.6	16.6	83.3
Controls	0	44.4	55.5	22.2	77.7
−4	T/T	T/TT	TT/TT	T	TT
GIP-AIMAH	55.5	44.4	0	22.8	77.8
Controls	44.4	55.5	0	27.8	72.2
+102	TT	TC	CC	T	C
GIP-AIMAH	0	0	100	0	100
Controls	0	0	100	0	100
+895	NN	N/insG	InsG/insG	N	InsG
GIP-AIMAH	0	0	100	0	100
Controls	0	0	0	0	100

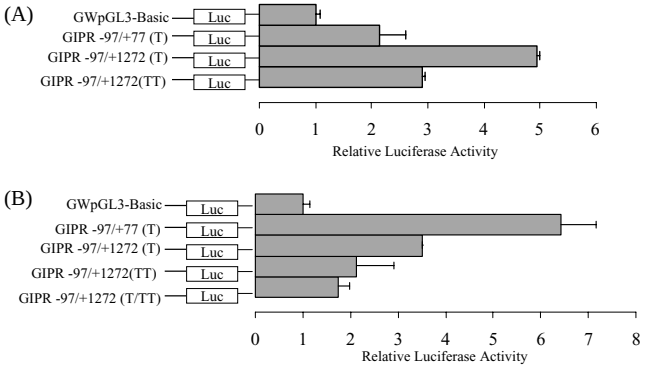

Fig. 2. Transcriptional activity of the GIPR-Luc chimeric genes in mouse Y1 cells and rat GC cells; analysis of two polymorphic variants. Chimeric constructs, containing two polymorphic variations of the human GIPR gene 5′-flanking sequence were co-transfected with pCMV-β-gal in Y1 and GC cells. After incubation for 48 h, luciferase and β-galactosidase activities were measured. The data represent the mean activity ± S.E. of two different transfections in duplicate; data are normalized to the activity of the promoterless construct, GWpGL3-Basic, after correcting differences in transfection efficiencies by the measurement of β-galactosidase activity. GIPR-Luc chimeras are shown on the left side, adjacent to the relative activity of the chimeric genes on the right side. (A): Y1 cells; (B): GC cells.

while the other four were heterozygotes and presented a T insertion (T/TT). The presence of a homozygous double T, which is the sequence deposited in the GenBank database, was not found in any patient. Sequencing of the genomic DNA of nine controls revealed almost the same genotypic distribution (T/T = 44.5% and T/TT = 55.5%). In human embryonic kidney cells (293HEK) and also in human adrenal H295R cells, the sequencing of this region revealed the presence of only one T.

At nucleotide +102, a homozygous T to C substitution was found in all nine patients as well as in all controls and HEK293 cells; at position +895 a G insertion was also found in all patients and controls.

3.2.3. Functional characterization of −4/−5 bp T/TT alleles

The possible effect of this nucleotide variation on the transcriptional activity of the GIPR gene promoter was assessed by transient transfection assays. Two GIPR-Luc chimeric plasmids were constructed using the promoterless report plasmid GWpGL3-Basic. Each construct (−4 T and −4 TT) contained −103 bp of the proximal pProm region and the first exon and intron (1271 bp) of the GIPR gene placed upstream the firefly luciferase gene. Transcriptional activity was determined by assaying the luciferase activity in transfected GC and Y1 cells. Transfection of both GIPR-Luc constructs resulted in high levels of luciferase activity when compared with pGL3-Basic (Fig. 2). The sequence containing a single T presented a 3.8- and 5.0-fold increase in activity, in mouse GC and rat Y1 cells, respectively. The sequence containing the double TT lead to a weaker increase; 2.8- and 2.9-fold increase, in GC and Y1 cells, respectively. Comparing the effect of these variants, we can see, in both cell lines, that the presence of TT confers 40% less of transcriptional activity when compared with the presence of only one T; co-transfecting T/TT did not prevent the decrease in activity.

4. Discussion

To determine whether genetic alterations in the putative regulatory region of the hGIPR gene could explain the tissue-specific adrenal overexpression of this receptor in GIP-dependent CS, we analyzed an extensive putative regulatory region of the GIPR gene in nine GIP-dependent adrenal tissues as well as in controls. Investigation of this region revealed the presence of 11 nucleotide variations not previously described. We analyzed both a proximal region and also a large distal upstream region, since it has been suggested that *cis*-acting distal regulatory elements may play a role in tissue-specific expression of the GIPR in rats [38]; we also studied the untranslated region downstream the TSS, since regulatory elements may exist in this region. Very close to the TSS, we found a frequent nucleotide variation; at position −4/−5, five patients presented the sequence ag***t***ggaGGC, but in four patients there was the presence of a heterozygous variation ag***t***ggaGGC/ag***tt***ggaGGC. The sequence deposited in the GenBank is ag***tt***ggaGGC, but in human embryonic kidney cells (HEK293) and in human adrenal cancer cell line 295R (H295R) the sequence found was ag***t***ggaGGC; in DNA from normal pancreas, the sequence found corresponds to the heterozygous ag***t***ggaGGC/ag***tt***ggaGGC. When we analyzed genomic DNA from controls, the same genotypic distribution was found. The investigation of possible effect of these variants in the transcriptional activity of the GIPR promoter was examined by luciferase activity of the promoter in rat somatomammotrophic GC cells and in mouse adrenal Y1 cells. Interestingly, in both cell lines, the sequence containing TT showed about 40–45% less transcriptional activity when compared to the sequence containing T. When we co-transfected T/TT, to mimic a heterozygous state, the negative effect of double TT persisted. It remains to be studied if this effect persists using a homologous system. The in vivo significance of this finding remains to be determined, but as the frequency found in patients was almost the same as in the controls, it unlikely to contribute to the pathogenesis of GIP-dependent CS.

The eight nucleotide variations found in the distal region of pProm of the GIPR gene are likely to be SNPs with no relation with GIP-dependent CS, since none was exclusively found in patients or in controls; all but one (A-5142T) occurred at similar frequencies in the two groups. Three frequent polymorphisms were located within *Alu* sequences, which have usually a high rate of nucleotide variations. It can also be noted that only two polymorphisms were located within possible CpG islands, but none of these belong to a CG dinucleotide, which could theoretically have

some influence on the methylation status of the promoter. Regarding other nucleotide differences found in our series, the homozygous T to C substitution at nucleotide +102 and an insertion of G at +895, both in intron 1, were found in 100% of patients and controls; this could represent a population variation or it may represent simply a mis-annotation in the GenBank.

So far, no mutations or polymorphisms have been described in the region upstream of the TSS and eight polymorphisms were described in the exons/introns of the hGIPR gene (dbSNP/ NCBI-ftp:/ftp.ncbi.nih.gov); five of them in coding regions (codons 170, 198, 207, 262, and 354) with four leading to amino acid changes but no association between GIPR polymorphisms and disease has been found [43].

Another possibility, yet to be examined, could be that changes in the methylation status in the promoter of the GIPR gene or in some regulatory gene are involved in its aberrant expression. Methylation of CpG island in the promoter is a powerful means of repressing expression [44,45]. In adrenal tumors, the association between H19 promoter methylation with the expression of IGF-II gene is well known [46]. The analysis of the GIPR gene revealed the presence of some potential, although small, CpG islands, one in the proximal pProm, three in the distal region, and also two localized downstream of the TSS. Future studies will be necessary to investigate if changes in the methylation status in regulatory elements of this gene or in other regulatory genes could be implicated in this condition.

Although larger population genotype studies may be necessary, our study indicates that proximal promoter rearrangements or mutations/SNPs in the regulatory region of the GIPR gene are unlikely to underlie GIP-dependent CS. Further studies will now be necessary to identify the tissue-specific transcription factors involved in the regulation of the hGIP-R gene expression and their potential alterations, which could result in its ectopic adrenal expression in GIP-dependent CS.

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