The GIP/GIPR axis is functionally linked to GH-secretion increase in a significant proportion of *gsp*⁻ somatotropinomas

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Abstract

Objective: Glucose-dependent insulinotropic polypeptide receptor (*GIPR*) overexpression has been recently described in a proportion of *gsp*⁻ somatotropinomas and suggested to be associated with the paradoxical increase of GH (GH-PI) during an oral glucose load.

Design and methods: This study was aimed at linking the GIP/GIPR pathway to GH secretion in 25 somatotropinomas-derived primary cultures and correlating molecular with clinical features in acromegalic patients. Given the impairment of the GIP/GIPR axis in acromegaly, an additional aim was to assess the effect of GH/IGF-1 stimulation on GIP expression in the enteroendocrine cell line STC-1.

Results: Nearly 80% of *GIPR*-expressing somatotropinomas, all of them negative for *gsp* mutations, show increased GH secretion upon GIP stimulation, higher sensitivity to Forskolin but not to somatostatin analogs. Besides increased frequency of GH-PI, *GIPR* overexpression does not appear to affect acromegalic patients' clinical features. In STC-1 cells transfected with GIP promoter-driven luciferase vector, IGF-1 but not GH induced dose-dependent increase in luciferase activity.

Conclusions: We demonstrate that *GIPR* mediates the GH-PI in a significant proportion of *gsp*⁻ acromegalic patients. In these cases, the stimulatory effect of IGF-1 on GIP promoter support the hypothesis of a functional GH/IGF-1/GIP axis. Further studies based on larger cohorts and the development of a stable transgenic model with inducible GIPR overexpression targeted to pituitary somatotroph lineage will be mandatory to establish the real role of GIPR in the pathogenesis of somatotropinomas.

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Introduction

Acromegaly is a rare and progressive hormonal syndrome commonly due to a benign monoclonal GH-secreting pituitary adenoma (GH-sec PA) (1). Patients often have well-known systemic complications at diagnosis with increased morbidity and mortality (2) and reflecting the slow progression of the symptoms over a period of many years, and the clinical diagnosis is frequently delayed.

A biochemical diagnosis of acromegaly is made by quantifying serum IGF-1 levels and determining autonomous GH secretion by measuring its levels after

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a standard oral glucose challenge (OGTT). In patients with active disease, oral glucose fails to suppress serum GH levels to below 0.4 µg/L, and in approximately 30% of cases, it increases paradoxically (1).

With the aim of clarifying the pathogenesis of GH-secreting PAs, we recently focused our attention on a member of the G-protein-coupled receptors superfamily (GPCR) – i.e. the glucose-dependent insulinotropic polypeptide receptor (GIPR) (3). Once activated by GIP, which is secreted by the K cells of the duodenum in response to meals, GIPR transduces extracellular stimuli into intracellular responses by activating the cAMP pathway (4, 5). In physiological conditions, the glucose-dependent secretion of insulin in pancreatic β cells after GIP stimulation exemplifies this mechanism (6). When inappropriately expressed in the adrenal gland, the GIPR may instead result in the development of an adrenal tumor (7), disrupting cAMP homeostasis by altering the cascade normally triggered by ACTH. Consequently, this leads to increased cortisol secretion and the development of food-dependent Cushing's syndrome (FDCS) (8, 9).

We found that nearly 30% of GH-sec PA expressed GIPR at significantly higher levels than normal pituitary glands. All were *gsp* negative, thus suggesting that these two events might be mutually exclusive and perhaps parts of the same pathogenic mechanism involving cAMP (3). Correlation of molecular findings with clinical data revealed that in most cases, GIPR overexpression was associated with a paradoxical increase in GH after OGTT (GH-PI) (3, 10). Similar to what is observed in FDCS, in GIPR-overexpressing GH-sec PAs, GH is inducible by meal (10), but unlike FDCS in which cortisol levels drop in parallel with GIP reduction; in acromegalic patients, the GH blood concentrations always remain above the diagnostic threshold. Although such observation might suggest that GIPR may not be the primary cause of acromegaly (10), these patients show abnormally high fasting and postprandial plasma GIP levels (11); this may be the consequence of a direct effect of GH, IGF-1 or both on GIP secretion.

Based on these premises, we aimed to study if a link between the GIP/GIPR axis and GH induction in GH-secreting PAs primary cultures exists and to GH-PI observed in acromegalic patients. Secondly, taking advantage of the GIP-secreting enteroendocrine model cell line STC-1, we assessed the effect of GH/IGF-1 stimulation on GIP secretion. Clinical-to-molecular correlation among patients from the current and our previous series (3) is also reported.

Subjects and methods

Patient tissues and primary cell cultures

We studied 25 consecutive active acromegalic patients (15 females; age 17–70 years, mean age 44 ± 15 years) diagnosed at the Endocrinology Unit of Padova University/Hospital and at the Pituitary Unit, Department of Neurosurgery, San Raffaele Hospital in Milan on the basis of consensus criteria (12). OGTT was performed in all 25 patients. An arbitrary threshold of greater than 20% of GH increase during this test – to discriminate between real increase and test variability (see below) – was set to differentiate paradoxical from non-paradoxical responders. Patients' clinical and hormonal features are reported in Supplementary Table 1 (see section on supplementary data given at the end of this article).

All 25 acromegalic patients underwent trans-sphenoidal surgery. Portions of the surgically removed specimens were fixed in 10% buffered formalin and then embedded in paraffin; standard sections stained with hematoxylin and eosin were used for diagnosis, whereas the presence of pituitary hormones was evaluated by standard immunocytochemical analyses. A second fragment for each tissue specimen was immersed in RNAlater (Ambion), kept at 4°C for 24 h and then stored at –20°C until RNA extraction. The remainder of each somatotropinoma was transferred to sterile cold complete culture medium and processed within 36 h as previously described (13). Informed consent was obtained from each patient. The study was conducted in accordance with the Helsinki Declaration.

Primary cells treatments and GH measurement

The effect of GIP and somatostatin analogs (SA) on GH secretion was examined in somatotropinoma-derived primary cells. Novartis Pharma AG kindly provided the SA Pasireotide (SOM230) and Octreotide (OCT), whereas GIP and Forskolin (FK) were purchased from Sigma-Aldrich.

After seeding and incubation of primary cells at 37°C for 48–60 h, the medium was removed and replaced with 2% FBS DMEM containing GIP (100 nM), FK (10 µM), OCT (100 nM) or SOM230 (100 nM). Cells were incubated for further 6 h (GIP and FK) or 24 h (OCT and SOM230) before the medium was recovered and frozen. GH was measured by Immulite 2000 (DPC, Carpinteria, CA, USA) (coefficient of variation (CV) <7%, lower detection limits 0.01 µg/L).

Immunofluorescence

Immunofluorescence for GIPR expression was performed on GH-sec PAs on conventional sections after deparaffinization in xylene, rehydration through graded alcohols to water and antigen retrieval (Dako) in 10 mM sodium citrate buffer (pH 6.0) for 10 min at 96°C. Sections of a normal human pancreas and of a GH-sec PA incubated with non-immune serum were used as positive and negative controls respectively.

GIPR expression was visualized with a rabbit polyclonal antibody (kindly provided by Prof. Timothy Kieffer, University of British Columbia, Vancouver, Canada; O/N, 4°C, 1:250) and Alexa Fluor 594-labeled donkey anti-rabbit IgG secondary antibody (Life Technologies, 1:250). Tissue sections were also stained for GH by incubating with a mouse anti-GH monoclonal antibody (Santa Cruz Biotechnology, 1:200) and Alexa Fluor 488-labeled donkey anti-mouse IgG secondary antibody (Life Technologies, 1:250). Cell nuclei were stained with 1.5 µg/mL Hoechst 33258 (Sigma-Aldrich). The cover slips were rinsed twice in excess PBS and mounted with Fluorescent Mounting Medium (Dako, Cat. No. S3023). The preparations were examined with a DMI6000CS fluorescence microscope (Leica Microsystems CMS) with a 63×/1.40 oil-immersion objective. Images were acquired by means of a DFC365FX camera and analyzed with Leica LAS-AF 3.1.0 software.

Nucleic acid isolation, cDNA synthesis and mutation screening

The tumor specimens collected in RNAlater were homogenized in a TissueLyser (Qiagen) in 1 mL of TRIzol reagent (Invitrogen) using a modified TRIzol protocol (14). RNA and DNA yields were determined on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity was tested with the Agilent 2100 Bioanalyzer (Agilent Technologies). Genomic DNA in the RNA was removed by DNAse, treating total RNA with Turbo DNA free kit (Ambion). RNA (500 ng) was reverse-transcribed with M-MuLV Reverse Transcriptase RNase H- (Euroclone, Pero, Italy) according to the manufacturer's recommendations.

Somatotroph adenomas were screened for somatic *gsp* mutations as reported previously (3). In nine GIPR overexpressing cases – included in this and previous cohort (3) – of which we had both somatic and matched germline DNA, the presence of somatic mutations in *GIPR* promoter (15) and transcribed regions has been evaluated. Lymphocytes DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA sequencing was performed using the BigDye 1.1 Termination Chemistry on an ABI 3730XL (Applied Biosystems). All primer sequences and PCR conditions are available upon request.

Quantitative RT-PCR (RT-qPCR)

RT-qPCR experiments were performed according to the MIQE guidelines. The GoTaq Probe qPCR Master Mix (Promega) and TaqMan Gene Expression Assays for the *GIPR* (Hs00609210_m1, Life Technologies) were used in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). *GIPR* flanking genes *GPR4* (NM_005282; For 5′-agggaacataagaccgcaat-3′; Rev 5′-tgcccttcacttgagttctg-3′), *EML2* (isoforms NM_001193268, NM_001193269, NM_012155 and NR_034098; For 5′-atgctgtcatcccacaagaa-3′; Rev 5′-ttggcaaagttcacctgttt-3′), *SNRPD2* (NM_004597; For 5′-ggagtgaacggagagcgtag-3′; Rev 5′-tcttattgttgcggcagttg-3′) and *QPCTL* (isoforms NM_001163377 and NM_017659; For 5′-acctgctgcagtctcatcc-3′; Rev 5′-tgagatggagcacgggta-3′) were assessed with the GoTaq qPCR Master Mix (Promega). Similarly, to assess the possible duplication of the *GIPR*-containing locus, two different genomic regions (CNV1, AC006132 For 5′-gtggcgggttgtgatgga-3′, Rev 5′-ccctccccttgccgattg-3′; CNV2, AC007191 For 5′-cgggtagggggagaacca-3′, Rev 5′-ctcgcccctctcctgaga-3′) were assessed by qPCR as previously described (16). For this purpose, tumoral DNA was extracted with a DNeasy Blood and Tissue Kit (see previously). A final concentration of either 300 nM (*GPR4*, *EML2*, CNV1 and CNV2) or 50 nM (*SNRPD2* and *QPCTL*) for both forward and reverse primers was used. All samples were tested in duplicate in a MicroAmp 96-well reaction plate sealed with an optical adhesive film (Applied Biosystems) with a proper amount of template (5–20 ng of cDNA or 10 ng gDNA) in 20 µL of reaction mixture. For *GIPR*, the reaction was set up using 20 ng of template. No-template controls were included in each run. The PCR conditions were 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Data were analyzed with SDS rel.2.4 (Applied Biosystems), with an automatically set baseline and a fluorescence threshold adjusted for measuring quantification cycle (Cq) values. Validation experiments performed using the standard curve method with five serial dilutions of genomic DNA from control subjects showed identical amplification efficiencies (100% ± 10%) calculated according to: $E = 10^{1/-\text{slope}} - 1$, for all assays. The amount of each target gene relative to

pituitary adenomas' most stable housekeeping gene, *HMBS* (14) for RT-qPCR and to a reference locus on chromosome 19 (*GPR4* gene, see above) for qPCR was ascertained by the ΔΔCq method. The threshold for distinguishing high- (GIPR-H) and low-expressing *GIPR* (GIPR-L) specimens was established previously (3).

Cell lines, transfection and dual-luciferase assay

The possible interaction between the GH/IGF-1 axis and the synthesis and/or secretion of GIP was investigated with murine enteroendocrine cell lines STC-1 (ATCC CRL-3254). Cells were cultured at 37°C and 5% CO_2 in DMEM-low glucose (ECM0749, Euroclone) supplemented with 10% FBS, 3.7 g/L $NaHCO_3$, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. Twenty-four hours before the experiment, STC-1 cells (1.75×10^5 cells/well) were seeded into 12-well plates. Cells were transiently transfected with 2 µL of Lipofectamine 2000 (Invitrogen) together with 1.6 µg of total DNA consisting of hGIP2.9kb-luc (a kind gift from Prof. T Kieffer generated by cloning a 2.9-kb fragment of human *GIP* promoter (–2844 to +57 bp) upstream luciferase gene (17)) and pRL-TK (Promega) and incubated for 24 h. Cells were then treated for further 24 h with GH (from 1 to 100 ng/mL), IGF-1 (from 0.1 to 100 ng/mL), insulin (100 nM) or the combination of FK and IBMX (10 µM each) and the effect on *GIP* promoter activity was examined. All compounds used for cell treatments were purchased from Sigma-Aldrich.

Cell media were removed, proteins were harvested in passive lysis buffer (Promega) and the relative luciferase activity was measured with the Dual-Luciferase Reporter Assay System and a GloMax 20/20 luminometer (Promega), according to the manufacturer's instructions.

As part of the study, RT-PCR was used to detect the presence of GH (*GH-R*) and IGF-1 receptors (*IGF-1R*) in STC-1 cells. RNA was extracted and reverse-transcribed as reported previously. Twenty-five nanograms of cDNA was amplified for 40 cycles with Taq G2 Flexi DNA Polymerase (Promega) and 5 pmol of sense and antisense primers, common to all known isoforms (*GH-R*, Primer F 5′-ggtcttcttaaccttggc-3′, Primer R 5′-tcttgcagcttgtcgttg-3′, *IGF-1R*, Primer F 5′-atggagtgctgtatgcttctg-3′, Primer R 5′-cggttcatggtgatcttctctc-3′). Mouse liver cDNA was used as a positive control for *GH-R* and *IGF-1R* expression.

Statistical analysis

For cell culture studies, all experiments were performed at least twice, and results are presented as the mean ± S.D. of at least triplicate determinations. Significance was determined by Student's *t*-test. To correlate clinical with molecular and biochemical data proportions and rates for categorical variables, means and standard deviations or medians and inter-quartile ranges (IQR) for parametric or non-parametric variables were calculated. Groups were compared with the chi-square test for categorical variables (or Fisher's exact test when the cell count was <5) or the Mann–Whitney test for quantitative variables, as appropriate. The SPSS 17 software package (SPSS) was used for all analyses. The significance level was set at $P < 0.05$ for all tests.

Results

Clinical and molecular analysis

Tumor specimens have been evaluated for *GIPR* expression by RT-qPCR. Based on our previous observations and established criteria (3), samples were divided into two distinct subgroups. The first comprises 15 samples expressing *GIPR* within the range for normal pituitaries (GIPR-L; from 1.6×10^{-4} to 1.7×10^{-2}, mean $2.9 \times 10^{-3} \pm 4.6 \times 10^{-3}$), and the second comprises 10 samples expressing *GIPR* at significantly higher levels (GIPR-H; from 0.02 to 0.27, mean 0.13 ± 0.08) (Fig. 1). By immunofluorescence we confirmed the high GIPR expression in the adenoma sections in the latter cases, both membrane and cytoplasmic immunoreactivity (3). Co-localization of red and green staining in the same cell confirmed that GIPR is expressed in GH-secreting tumor cells (Fig. 2).

The thorough assessment of clinical features in the 25 acromegalic patients revealed that 8/10 GIPR-H patients showed a GH-PI (Fig. 3 and Supplementary Table 1). The remaining two patients showed a decrease of GH that did not fall below 0.4 µg/L (#6) or a slight increase not exceeding the previously established cut-off of 120% (#9). In contrast, only 3/15 GIPR-L patients (#5, #7 and #15) showed a GH-PI. Among GIPR-H subjects with a postoperative OGTT, one was cured by adenomectomy and the paradoxical GH secretion was abolished. In three further cases, despite evidence of a markedly reduced nadir GH in two of them, persistence of GH levels above 0.4 µg/L confirmed the persistence of the diseases (Supplementary Table 1); the GH-PI was also maintained.

Seeking the molecular cause of *GIPR* gene overexpression, we studied the *GIPR* locus in GIPR-H tumors. On sequence analysis, no somatic mutations were identified. To evaluate a possible impairment of

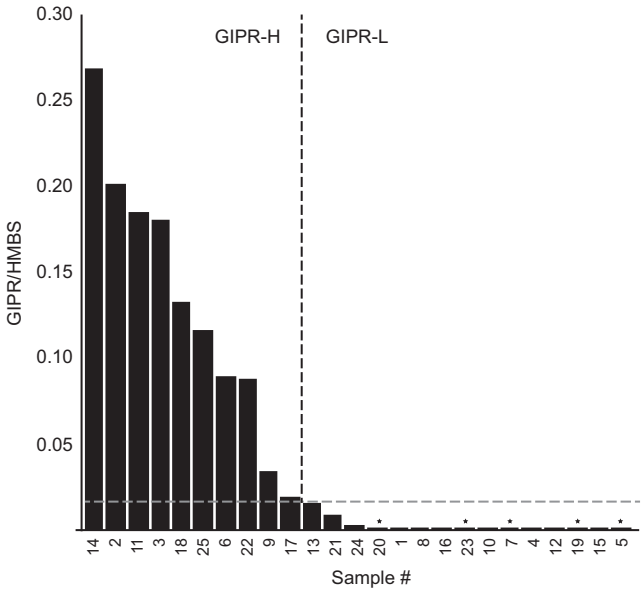

Figure 1

GIPR mRNA steady-state level in tumor tissue samples. *GIPR* mRNA levels were normalized against the most stable pituitary housekeeping gene, HMBS. Patients' identification numbers have been reported in Supplementary Table 1. The dashed gray line represents the threshold above which samples were classified as overexpressing *GIPR* (see 'Material and methods' section). The dashed black line divides the chart in two parts that include GIPR-H (left) and GIPR-L (right) samples. §*gsp*⁺.

transcription involving the 19q13.32 locus (SF 1a), we compared the steady-state level of *GIPR*'s upstream (*GPR4* and *EML2*) and downstream flanking genes (*SNRPD2* and *QPCTL*) mRNA between six GIPR-H and ten GIPR-L samples. No significant difference emerged between the two groups (see SF 1b). In addition, copy number gains of *GIPR* were excluded by qPCR testing two different genomic regions (CNV1 and CNV2 in SF 1a) in two GIPR-H and five GIPR-L specimens respectively (SF 1c).

Tumor specimens were then further evaluated for *gsp* mutations. As shown in Fig. 1, one of two mutations was identified in 5 of the 25 cases, and only associated with low *GIPR* expression. In all but one case with an A/T transition at codon 227 (Q227L), the mutation occurred at codon 201 (three R201C and one R201H).

Primary cell treatment and GH measurement

To definitively confirm the link between GIP/GIPR and the GH-PI, somatotropinoma-derived primary cultures have been established and cells were treated with GIP.

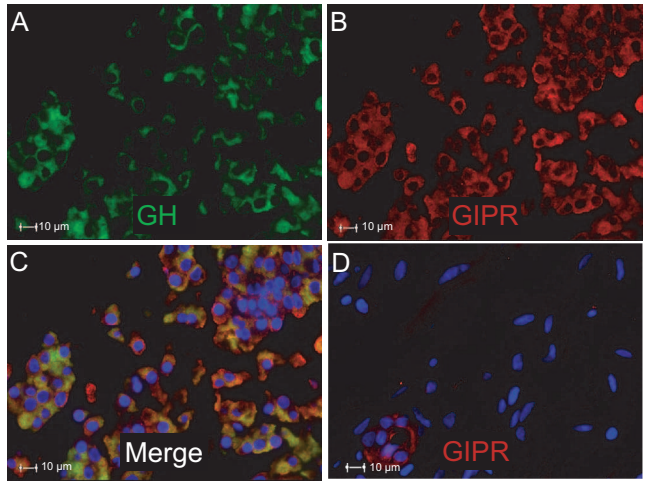

Figure 2

Representative immunofluorescence staining of a GH-sec PA with high GIPR expression. (A) GH is visualized in the green immunofluorescent channel, whereas (B) GIPR in the red one. (C) Co-localization of red and green staining in the same cell confirms that GH-secreting tumor cells express GIPR. Cells are counterstained with Hoechst (blue) to mark nuclei. (D) Positive control is a section of a normal human pancreas. Immunofluorescent images are at 100× magnification.

As shown in Fig. 4A, 6/10 GIPR-H cases significantly responded to GIP stimulus (GH secretion mean induction 204%±95%). In the remaining four cases, two (#6 and #22) showed a GH increase – 121% and 137% respectively

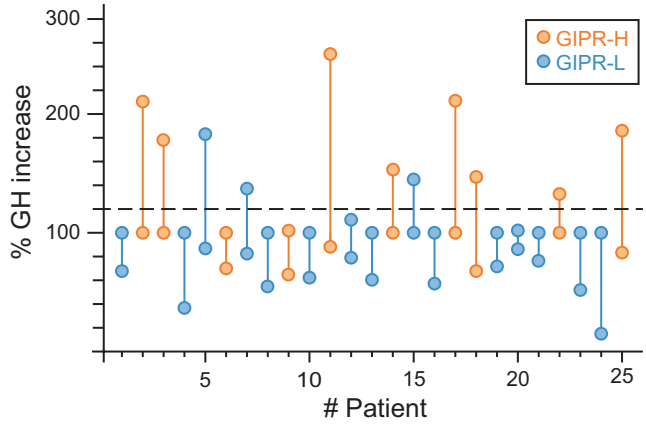

Figure 3

Zenith and nadir of two-hour OGTT in the cohort of acromegalic patients. Values are expressed as percentage of the zero time point. The dashed black line represents the 120% threshold we considered for defining an increase as paradoxical.

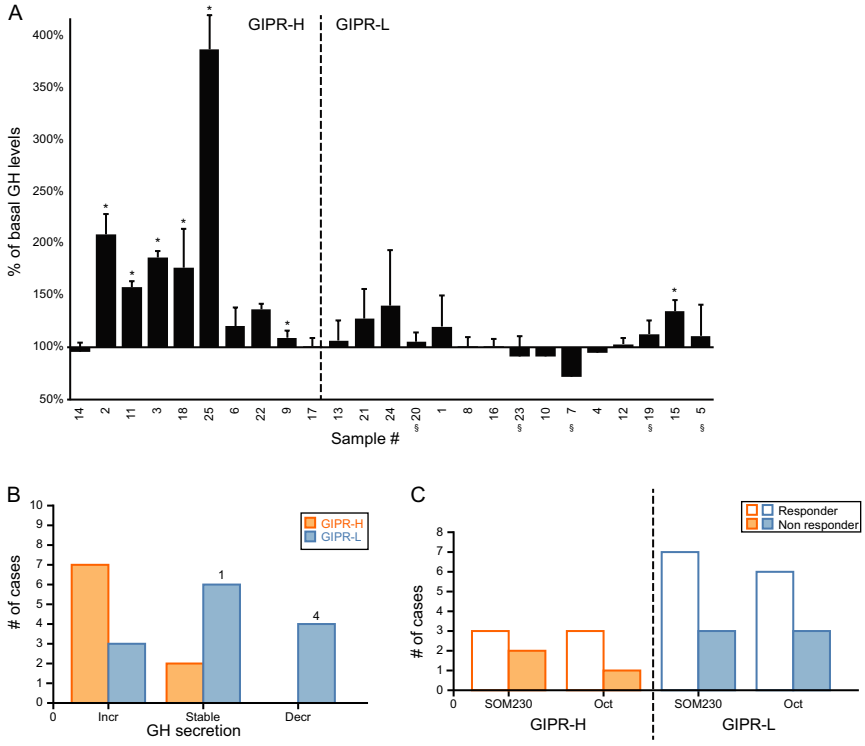

Figure 4

GH-sec PA-derived primary cultures responsiveness to different stimuli. (A) Cell cultures have been treated with 100 nM GIP or vehicle and GH secretion has been evaluated. The level of GH secretion was expressed as relative percentage to vehicle-treated tumor cells. §*gsp*⁺, *$P<0.05$. Samples have been ordered according to Fig. 1 to easily compare *GIPR* expression data with functional *in vitro* studies. (B) Primary cultures responsiveness to FK (10 μM). Bars represent the number of samples that either showed a statistically significant increase (incr), a decrease (decr) of GH after FK treatment. Stable are those cultures in which GH secretion is not influenced by FK treatment. The number of *gsp*⁺ cases in each group, when different from 0, is reported above the correspondent bar. (C) Primary cultures responsiveness to SOM230 or Oct (100 nM) divided according to the *GIPR* expression profile. Responders are considered as those cases with a statistically significant decrease of GH secretion compared to the correspondent vehicle-treated cells.

– that did not reach statistical significance (due to high variability among vehicle-treated wells). In contrast, among GIPR-L primary cells, only in one case (#15) a modest increase of GH secretion after GIP stimulation was observed. *In vitro* GH increase due to GIP treatment and *GIPR* overexpression were positively correlated (Spearman's Rho $R=0.522$, $P=0.006$).

To characterize the activation status of the cAMP signaling pathway, according to *GIPR* expression, primary adenoma cells were treated with FK and GH secretion was evaluated. As shown in Fig. 4B, GIPR-H tumors responded more frequently (7/9 vs 3/13, $P<0.05$) and with higher response to FK (185%±113% vs 110%±63%, $P=0.05$) than GIPR-L adenomas. Interestingly, in four of five *gsp*⁺ tumors, FK treatment induced a significant reduction of GH (mean decrease 44%±10%) possibly as a consequence of a non-inducible pathway (18).

Whenever possible, primary cultures were treated with either OCT or SOM230, the SSTR2- and SSTR5-selective agonists respectively. Both molecules were equally effective in inducing GH decrease in both GIPR-H and GIPR-L somatotropinomas (Fig. 4C).

Clinical-to-molecular phenotype

To increase the power to identify possible association, we then combined data from this and from a previously published cohort (3). This resulted in 47 cases (15 males) for which both molecular and comprehensive clinical data were available. The GH-PI was observed in 43% (20/47) of acromegalic patients and is strictly associated with *GIPR* expression in somatotropinomas but not to *gsp* mutational status. The group of 20 patients with a GH-PI included 81% (13/16) and 23% (7/31) of GIPR-H

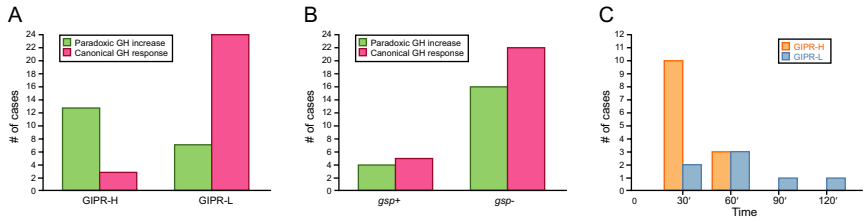

Figure 5

Correlation between biochemical and molecular observations in acromegalic patients. Subjects have been grouped according to (A) *GIPR* expression profile or (B) *gsp* mutational status. Bars represent the number of patients within each group presenting a paradoxical or a canonical response of GH during OGTT. In panel (C) the bars heights correspond to the number of patients with a paradoxical increase of GH observed that begin at the time point indicated in the *x*-axis during OGTT.

and GIPR-L adenomas respectively (Fig. 5A, Pearson χ^2 13.75, $P<0.001$). In contrast, a frequency of ~40% among paradoxical acromegalic patients could be observed in *gsp*⁺ (4/9) and *gsp*⁻ (16/38) (Fig. 5B).

Although patients with GIPR-H tumors tended to be males (50% of males vs 27% of females, $P=0.18$) with higher serum GH levels (26.8±29.4 vs 17.2±15.9, $P=0.159$), these differences were not statistically significant. In addition, no differences were observed in the age at diagnosis, basal plasma PRL levels or in the distribution of macro and micro adenomas between GIPR-H and GIPR-L groups. We then evaluated the initial GH increase in patients with GH-PI according to the somatotropinoma *GIPR* expression status. In 77% (10/13) of patients with a GIPR-H tumor, GH increase occurred very early during OGTT (i.e. within 30 min), whereas only in one-third of patients with a GIPR-L adenoma (2/7) ($P=0.06$, Fig. 5C).

Cell lines, transfection, dual-luciferase assay

To test if the high levels of circulating GIP reported for acromegalic patients (11) might be a direct consequence of high GH or IGF-1, GIP promoter inducibility was assessed in the murine enteroendocrine cell line STC-1. hGIP2.9kb-luc-transfected STC-1 cells were treated with increasing concentrations of either IGF-1 or GH, and luciferase activity was determined. A 100 nM insulin treatment was used as positive control.

As reported in Fig. 6A, the IGF-1 treatment induced a dose-dependent increase in luciferase activity with a significant effect already at 0.1 ng/mL ($P<0.05$). The maximum increase (6.4-fold±0.5, $P>0.001$) was reached at 50 ng/mL, comparable with that generated by IBMX+FK used as second positive control of *GIP* promoter activation. In contrast, no induction of the *GIP* promoter activity was seen at any concentration of GH. These data are consistent

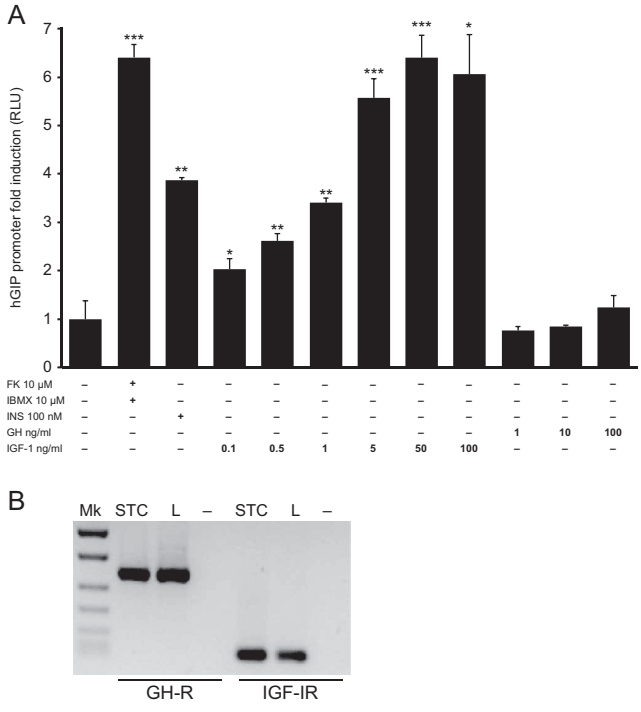

Figure 6

Effect of GH, IGF-1 and insulin on GIP promoter activity in STC-1 cells. (A) STC-1 cells were transfected with the GIP-luc reporter plasmid (hGIP2.9kb-luc), and luciferase expression was measured in the presence of increasing concentrations of GH or IGF-1 or of insulin (100 nM) or a combination of IBMX+FK (the two latter are positive control of GIP-luc induction). The relative activity was adjusted for transfection efficiency using pRL-TK. Results are expressed as relative luciferase activity compared with basal level of GIP-luc-transfected STC-1 without treatments. Error bars represent standard deviation of the mean. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with cells given no drug treatment (*t*-test). All experiments were performed at least in triplicate. (B) IGF-1 and GH receptor expression in STC-1 cells (STC). L mouse liver, Mk marker, – negative control.

with the observation that IGF-1R is expressed in STC-1 cells (Fig. 6B).

Discussion

Recently two studies – one by our group – raised the hypothesis that GIP/GIPR could promote the GH-PI in a proportion of acromegalic patients (3, 10) by counteracting hypothalamic inhibitory activity induced by glucose (19, 20). In the current study, we confirmed that impaired expression of a functional GIPR, rather than an altered hypothalamic somatostatinergic activity (21), mainly contributes to this effect. Indeed, previous observations of functional coupling of GIPR with Gαs in GIPR-transfected GH3 cells (3), and the current findings of GIP responsiveness of most high *GIPR*-expressing primary cultures, strongly suggest that in about 30% of GH-sec PAs, activation of an unconventional GIP/GIPR axis, after an oral glucose administration, stimulates GH secretion by mimicking the cellular events triggered by GHRH stimulation (22, 23, 24). The co-localization of GIPR and GH in somatotroph tumor cells further supports the assumption of a mechanism of action that parallels the GIP-mediated cortisol synthesis and secretion in FDCS patients (8).

In nearly 20% of cases, however, GH plasma levels increase after glucose stimuli in spite of low *GIPR* levels and vice versa – i.e., high *GIPR* level without GH-PI. Given the high specificity that characterizes the GIP/GIPR binding (25), in the former cases, GH-PI is unlikely caused by GIP binding to alternative GPCRs, which could instead be directly activated by a gastroenteropancreatic hormone (e.g. glucagon-like peptide-1). The secondary activation by an hypothalamus-mediated mechanism after an hyperglycemic stimulus (e.g. GnRH (26)) may represent an alternative hypothesis to explain this phenomenon, being a functional GnRH receptor identified in nearly 15% of *gsp*⁻ GH-sec PA (27). Looking to paradoxical cases, the evidence that in GIPR-negative patients GH increase occurs later during the test compared to GIPR-positive suggests, in addition, that a GPCR-independent mechanism cannot be completely ruled out and that the GH-PI may occur when hyperglycemia cannot abolish the endogenous GHRH-induced GH rise, a mechanism proposed for explaining the GH-PI observed in 8% of normal subjects (34). On the other hand, neither *GIPR*-inactivating mutations nor nucleotide variants (i.e. SNP rs1800437) associated to enhanced receptor desensitization (28) explain GIP unresponsiveness in GIPR-H cases. Although technical reasons could not be fully ruled out, mechanisms affecting GPCR modulation, involving β-arrestins, GPCR kinases or regulators of G-protein signaling, as already associated to SSTRs activation (29), may represent an intriguing alternative.

Besides an higher incidence of GH-PI, as in FDCS (30) the presence of GIPR overexpression does apparently not influence the major clinical features in acromegalic patients as already reported in a subset of the present cohort (3). This partially reflects the results of previous studies in which patients' clinical features have been analyzed with respect to *gsp* mutational status. Besides a slightly higher responsiveness to SA (31) and a more common small size and densely granulated pattern reported for *gsp*⁺ tumors, no relevant difference in clinical features (e.g. GH/IGF-1 levels, disease duration, cure rate and outcome) have been found (32, 33). This may imply that although the improper cAMP pathway activation represents a compelling mechanism for explaining GH cell hypersecretion, its clinical significance has not been clearly defined.

GIPR-overexpressing GH-sec PAs respond more frequently and effectively to the adenylate cyclase activator FK by increasing GH secretion, in accordance with previous observations of a less inducible cAMP-signaling pathway in *gsp*⁺ tumors (18). The exclusion of these latter from our cohort to specifically determine the sole contribution of GIPR to this, however, greatly reduces the cohort size and prevents us from drawing final conclusions on this aspect, despite a tendency toward a higher responsiveness to FK in GIPR-H compared to GIPR-L/*gsp*⁻ emerges. Evaluation of a larger cohort would be desirable to allow a reliable subgroup analysis. Given the correlation between *GIPR* overexpression and GH-PI, a possible relationship between *GIPR* mRNA steady-state level and clinical features could be indirectly inferred, thus stratifying acromegalic patients according to OGTT response. To our knowledge, no studies investigating the possibility of such an association have been performed, except for one showing that the simultaneous presence of a GH paradoxical response to TRH and the lack of partial GH inhibition after OGTT were related to smaller tumor size and postoperative long-term control of the disease (21).

Activating mutations in the gene promoter (15) or transcribed regions are unlikely to underlie *GIPR* overexpression in GH-sec PAs. This resembles FDCS tumors (34, 35) that does not even present changes in the level of transcription factor – e.g. Sp1/Sp3, Pax6 and Foxa1/Foxa2 (34, 36, 37) – involved in *GIPR* expression

regulation (38, 39, 40). Based on the quantification of flanking gene expression and *GIPR* copy number, we can also exclude a general (40) or locus-specific impairment of transcription as well as of a gross chromosomal duplication. A recent large integrated epigenomic and transcriptomic study associated a *GIPR* locus methylation defect to gene expression impairment (41). Although this mechanism seems at the moment the most probable, alternative genetic events – e.g promoter swapping, gene fusion – need to be considered for definitely explaining this phenomenon.

Regardless of the reasons for this upregulation, these data together suggest that the aberrant expression of GIPR may be a secondary rather than a primary event. This has been recently reported in Cushing's disease and aldosteronomas in which EGFR or GnRHR overexpression is secondary to *USP8* deubiquitinase gene mutations (42) or to *b-catenin* constitutive active mutations (43) respectively. On the other hand, the observation that GIPR-altered expression may be sufficient to trigger adrenocortical tumor formation (7) and its mutual exclusivity with *ARMC5* gene mutations in patients with bilateral macronodular adrenal hyperplasia (16, 44) cannot exclude the possibility that GIPR overexpression might be the pivotal driver of tumor transformation in endocrine tissues.

In a previous report, in spite of the recognized analogies in the mechanism regulating the aberrant secretion of GH and cortisol in GIPR-expressing GH-sec PA and adrenal nodules respectively, aberrant activation of the GIP/GIPR pathway has been excluded as the primary cause of acromegaly as the GH blood concentration remains always above the diagnostic threshold (10). The abnormally high fasting and postprandial plasma GIP levels in acromegalic patients (11) led us to postulate that in GIPR-expressing PAs, GIP plasma levels could be high enough to chronically trigger adenylyl cyclase/cAMP signaling (3). In addition, the stimulatory effect of IGF-1 on *GIPR* promoter activity in STC-1 may suggest the possible presence of a self-sustaining GH/IGF-I/GIP axis in these patients. Food dependency indeed might characterize the first phase of the disease and prolonged exposure to high circulating GH levels might induce persistently elevated GIP levels that continuously trigger the adenylyl cyclase/cAMP signaling cascade in the GIPR-overexpressing GH-sec PA. Higher basal circulating GH levels should characterize a persistent cAMP stimulation – i.e. patients overexpressing GIPR – which we have seen only as tendency. On the other hand, a similar expectation could also be formulated for *gsp*⁺ samples for which, on the contrary, data are conflicting and inconclusive (33). This may imply that although important in tumoral transformation, as confirmed by recent NGS studies (45), an impaired cAMP pathway could not be used to predict basal serum GH levels.

In conclusion, we here demonstrate for the first time that in GH-sec PAs, the functional GIP/GIPR pathway mediates GH secretion and is frequently associated with GH-PI. Whether GIPR overexpression may be associated with a specific clinical or molecular phenotype in GH-sec PAs and acromegaly, and may thus be explored for novel therapeutic approaches, as recently proposed for neuroendocrine tumors (46), remains to be established. Finally, further studies based on a large number of cases and the development of a stable transgenic model with inducible GIPR overexpression targeted to pituitary somatotroph lineage will be required to establish the real role of GIPR overexpression in the pathogenesis of GH-sec PAs and to establish the possible existence of a GH/IGF-1/GIP axis in a proportion of acromegalic patients.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-16-0831.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

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