Somatostatin receptor subtype 5 (SSTR5) mRNA expression is related to histopathological features of cell proliferation in insulinomas

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Abstract

Insulinomas are rare endocrine neoplasias that constitute the most frequent islet cell tumours. Somatostatin (SST) analogs are tentatively used to inhibit insulin secretion and control tumour growth in patients with local invasion or inoperative metastasis, but variable responses have been reported. Data regarding somatostatin receptor (SSTR) subtypes expression in insulinomas are conflicting. In this study, we evaluated 16 cases of primary insulinomas (including four primary plurihormonal tumours) and two hepatic metastases. Histopathological and immunohistochemical analysis for some features associated with tumour aggressiveness and semi-quantitative RT-PCR for SSTR1-5 and real-time qPCR for SSTR5 were performed. SSTR subtypes 1, 3, and 5 were expressed in 100%, SSTR2 in 89%, and SSTR4 only in 22% of the insulinomas. SSTR5 mRNA was positively correlated with histopathological features related to tumour aggressiveness (large tumour diameter, well-differentiated endocrine tumour with uncertain behaviour and higher number of cells with nuclear atypia). SSTR5 mRNA expression in primary insulinomas was lower than in primary plurihormonal tumours ($P < 0.05$). The observed positive correlation between SSTR5 expression and tumour size suggests that the use of SST analogues more specific to SSTR5 in the treatment of insulinomas deserves attention.

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Introduction

Somatostatin (SST) action is mediated by five known SST receptors (SSTR) subtypes — SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5, members of G-protein-coupled membrane receptors family, with negative modulation of adenyl-cyclase (Patel 1999). The different subtypes are related to distinct cellular effects: SSTR5 seems to be involved in the control of

insulin and glucagon secretions; SSTR3, and to a lesser extent SSTR2, can induce apoptosis, and SSTR1 and 5 have inhibitory effects on the cell cycle, while the function of SSTR4 remains unknown (Lamberts *et al.* 2002).

Insulinomas are rare endocrine neoplasias that constitute the most frequent islet cell tumours, with an estimated incidence of four cases per one million people per year. Insulinomas are usually benign

(90% of cases), solitary (90%), intrapancreatic (99%) tumours (Service *et al.* 1991). The smaller tumours tend to be homogeneous without local invasion or distant metastasis. Insulinomas are generally composed of sheets of small round cells and mitoses are unusual. The amount of intervening stroma is extremely variable, and may appear dense and hyalinized. There is a well-organized relationship between the neoplastic cells and the numerous stromal vessels. Three histopathological patterns are observed in insulinomas: (a) a solid, diffuse pattern; (b) a ribbon-like, trabecular pattern and (c) an acinar or duct-like pattern. More than one of these architectural patterns may occur in the same neoplasm (Solcia *et al.* 1997).

Although the underlying mechanisms related to subtype selectivity have not been completely elucidated, SST analogues are used in the treatment of several tumours since they may inhibit hormonal secretion, induce apoptosis and affect cell proliferation. Octreotide and lantreotide are tentatively used to inhibit insulin secretion and control tumour growth in patients with local invasion or inoperative metastasis from insulinomas, but variable responses have been reported (Dogliotti *et al.* 2001). Several mechanisms may account for these variable responses such as receptor down-regulation, desensitization and absence of expression of SSTR subtypes with high affinity for the analogues in the tumour (de Herder *et al.* 2003). The relative frequency of SSTR expression in β -cells was determined to be SSTR1 (100%) > SSTR5 (87%) > SSTR2 (46%) > SSTR3 (28%) > SSTR4 (17%) (Kumar *et al.* 1999) while data regarding SSTR subtypes expression in insulinomas are conflicting. Reverse transcription polymerase chain reaction (RT-PCR) analysis performed in four series of insulinomas showed the following subtype frequency: SSTR1 = SSTR4 (100%) > SSTR2 (75%) > SSTR3 (50%) in 4 cases (Kubota *et al.* 1994); SSTR2 = SSTR4 = SSTR5 (100%) > SSTR3 (80%) > SSTR1 (60%) in 5 cases (Jais *et al.* 1997); SSTR1 (90%) > SSTR3 (84%) > SSTR2 (74%) > SSTR4 = SSTR5 (42%) in 19 cases (Papotti *et al.* 2002); SSTR2 = SSTR5 (70%) > SSTR1 (50%) > SSTR3 = SSTR4 (15–20%) in 20 cases (Bertherat *et al.* 2003). In 36 cases studied by immunohistochemistry, Kulaksiz *et al.* (2002) reported SSTR3 = SSTR5 (78%) > SSTR2A (58%) > SSTR1 (31%).

In this study, we evaluated the mRNA expression of the five SSTR subtypes in insulinomas by RT-PCR and of SSTR5 by real-time quantitative PCR (qPCR) correlating the results with histopathological and immunohistochemical data.

Materials and methods

Patients and tissue samples

From 1999–2003, tumour tissues were obtained from patients diagnosed with insulinoma, based on the clinical features of Whipple's triad of symptoms with concomitant endogenous hyperinsulinemia and positive immunohistochemistry of the tumour for insulin. During laparotomy surgery, tumour fragments were collected in sterile containers and frozen in liquid nitrogen. Eighteen insulinomas from 17 patients were included in the study: two liver metastases of insulinomas and 16 primary tumours, comprising one insulinoma from a patient diagnosed with Multiple Endocrine Neoplasia Type 1 (MEN1) syndrome, one sample corresponding to the primary insulinoma of one hepatic metastasis and 14 sporadic insulinomas. Most tumours were encapsulated and the fragments for RNA extraction were carefully removed from the central portion, in order to avoid contamination with normal pancreatic tissue. This study was approved by the Ethical Committee of Hospital das Clínicas of the University of São Paulo School of Medicine and informed consent was obtained from all patients. All laparotomies were performed by the same surgeon.

Histopathological and immunohistochemical analysis

Sections were histologically analysed by hematoxylin and eosin staining. Histopathological examination was performed based on nuclear atypia (coded as mild, moderate or severe), mitotic index (number of mitosis/10 high-power fields [HPF]), presence or absence of perineural and vascular invasion. Immunohistochemical staining was performed in paraffin embedded blocks by avidin-biotin peroxidase complex (ABC) method using anti-human chromogranin A antiserum (Biogenex Laboratories, San Ramon, CA USA), synaptophysin, insulin, SST, glucagon, gastrin, tumour protein p53 and proliferation-related Ki-67 antigen (Dako Cytomation Denmark A/S, Copenhagen, Denmark). The tumours were classified as: well-differentiated endocrine tumours with benign behaviour (WDET-BB), confined to the pancreas, non-angioinvasive, no perineural invasion, <2 cm in diameter, <2 mitoses/10HPF, and <2% Ki-67 positive cells; well-differentiated endocrine tumours with uncertain behaviour (WDET-UB), confined to the pancreas with one or more of the following features: ≥ 2 cm in diameter, 2–10 mitoses/10HPF, >2% Ki-67 positive cells, angioinvasion, perineural invasion; well-differentiated endocrine carcinoma

(WDEC), in the presence of gross local invasion and/or metastases; and metastasis of endocrine carcinoma (MEC) (Komminoth *et al.* 2004). All samples were evaluated by a single pathologist.

RNA isolation and cDNA synthesis

After tumour pulverization with a dismembrator (B. Braun Biotech International, Melsungen, Germany) at liquid nitrogen temperature, total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. To avoid possible genomic DNA contamination, all RNA samples were treated with RNase-free DNase (Promega). After phenol treatment and drying, RNA was dissolved in RNase-free water and RNA concentration was spectrophotometrically determined. RNA quality was checked by agarose gel electrophoresis.

Semi-quantitative RT-PCR

mRNA expressions of SSTR subtypes in this tumour series were performed by semi-quantitative RT-PCR. Total RNA (3 µg) was reverse transcribed at complementary DNA (cDNA) using SuperScript II Reverse Transcriptase and random primers (Invitrogen Life Technologies) according to manufacturer's recommendations and diluted with double distilled water to a final volume of 100 µl. PCR experiments were carried out in 25 µl final reaction volume containing 3 µl cDNA template, 0.5 µM sense and antisense of target gene primers, 0.25 µM sense and antisense of housekeeping gene primers (breakpoint cluster region [BCR] gene), 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2.0 mM MgSO₄, 0.2 mM deoxynucleotide triphosphates (dNTPs), and 1.0 U Platinum *Taq* High Fidelity (Invitrogen). PCR amplification for each gene studied was performed under the following cycling conditions: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at specific temperatures (55 °C for SSTR2 and SSTR4, 56 °C for SSTR5 and 58 °C for SSTR1 and SSTR 3) for 45 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. The number of cycles to achieve the exponential phase for PCR amplification was 35 for all SSTR subtypes co-amplified with BCR. The PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide and visualized by ultraviolet light. The band intensities of two independent experiments were analysed by Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA, USA). The mean expression levels of the target genes were normalized by the mean BCR housekeeping gene expression and the ratios of each gene expression

(target gene/BCR) were calculated in all samples as arbitrary units of optical density. Human SSTR genes are intronless, and only the primers for BCR amplification were so designed that PCR products comprised portions of two contiguous exons. The primers were designed with the primer3_www.cgi v 0.2 program (Rozen & Skaletsky 2000) as follows:

BCR GenBank (377-bp product):

Sense: 5'-GAGAAGAGGGCGAACAAAG-3'

Antisense: 5'-CTCTGCTTAAATCCAGTGGC-3'

SSTR1 GenBank (217-bp product):

Sense: 5'-TATCTGCCTGTGCTACGTGC-3'

Antisense: 5'-GATGACCGACAGCTGACTCA-3'

SSTR2 GenBank (281-bp product):

Sense: 5'-CAGTCATGAGCATCGACCGA-3'

Antisense: 5'-GCAAAGACAGATGATGGTGA-3'

SSTR3 GenBank (188-bp product):

Sense: 5'-TCAGTCACCAACGTCTACATCC-3'

Antisense: 5'-ACGCTCATGACAGTCAGGC-3'

SSTR4 GenBank (321-bp product):

Sense: 5'-ATCTTCGCAGACACCAGACC-3'

Antisense: 5'-ATCAAGGCTGGTCACGACGA-3'

SSTR5 GenBank (222-bp product):

Sense: 5'-CGTCTTCATCATCTACACGG-3'

Antisense: 5'-GGCCAGGTTGACGATGTTGA-3'

SSTR5 quantitative PCR analysis

Total RNA from 17 tumour samples were extracted using TRIzol reagent (Invitrogen) and treated with RNase-free DNase (Promega) as described above. Transcript levels of SSTR5 was determined as the number of transcripts relative to those of housekeeping gene proteasome 26S ATPase subunit 6 (PSMC6). The primers designed with the primer3_www.cgi v 0.2 program (Rozen & Skaletsky 2000) were follows:

PSMC6 GenBank (196-bp product):

Sense: 5'-GCTGCGTCCAGGAAGATTAG-3'

Antisense: 5'-TGCGAACATACCTGCTTCAG-3'

SSTR5 GenBank (156-bp product):

Sense: 5'-GTGACAACAGGACGCTGGT-3'

Antisense: 5'-TGGTGACGGTCTTCATCTTG-3'

qPCR analyses of SSTR5 were performed in the Rotor-Gene RG-3000 (Corbett Research, Sidney, Australia) using Quantitect SYBR Green RT-PCR for quantitative, real time, one step RT-PCR (Qiagen GmbH, Hilden, Germany), according to the instructions provided by the manufacturer. Reactions lacking reverse transcriptase were also run to generate controls

Table 1 Clinical and laboratory data of patients with diagnosis of insulinoma. Age and duration of symptoms were represented in median, minimum and maximum values. All other variables were represented in mean $\pm$ s.d.

		Well-differentiated endocrine tumours			
		Benign behaviour (WDET-BB)	Uncertain behaviour (WDET-UB)	Carcinoma	
				Primary (WDEC)	Metastases (MEC)
All groups					
Clinical data					
Total of patients	17	9	6	1	2
Gender (M/F)	8/10	3/6	3/3	1/0	1/1
Age (years)	43 (14–79)	30 (14–71)	50 (22–79)	43	60 (43–77)
Duration of symptoms (months)	24 (1–168)	15 (3–48)	24 (1–156)	6	87 (6–168)
Laboratory data					
Glucose (mmol/l)	2.25 ± 0.19	1.96 ± 0.22	2.20 ± 0.34	3.28	3.16 ± 0.11
Insulin (pmol/l)	302.9 ± 69.26	244.1 ± 66.7	209.7 ± 61.2	930.0	533.7 ± 396.3
C-peptide (nmol/l)	1.78 ± 0.23	1.49 ± 0.26	2.28 ± 0.50	2.45	1.51 ± 0.94

WDET, well-differentiated endocrine tumour; BB, benign behaviour; UB, uncertain behaviour; WDEC, well differentiated endocrine carcinoma; MEC, metastases of endocrine carcinoma

for assessment of genomic DNA contamination. Reaction mixture consisted of 12.5 μ l of SYBR RT-PCR Master Mix, 0.25 μ l of QuantiTect RT Mix, 0.2 μ M sense/antisense primers and 5 μ l (20 ng/ μ l) of total RNA template. RNA template concentrations (100 ng/ μ l, 50 ng/ μ l, 25 ng/ μ l, 12.5 ng/ μ l, 6.5 ng/ μ l and 3.2 ng/ μ l) were used to generate a standard curve to evaluate the amplification efficiency of each target gene in comparison to PSMC6. The reaction was carried out under the following cycling conditions: 50 °C for 30 min for reverse transcription, heated to 95 °C for 15 min, and then cycled 35 times at 94 °C for 20 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. To verify the specificity of the amplification reaction, melting-curve analysis was performed using the following parameters: 72 °C ramping to 99 °C at 0.2 °C/second, in conjunction to an agarose gel electrophoresis to visualize the band for PSMC6 and SSTR5 with the correspondent size; 196 bp and 156 bp, respectively. Relative quantification was calculated using the mathematical model described by Pfaffl 2001.

Statistical analysis

Nonparametric tests were employed in all comparisons. Data were classified in Van der Waerden rank scores by ranking the data, divide by one plus the number of observations transformed to a normal score by applying the inverse of the normal distribution function (Hallin & Jurčková 1999). Spearman's ρ (rho) coefficient was calculated in all correlation analysis after normalization of the data by taking the z-scores

of rank of the values in increasing order subtracted by 0.5 and divided by the size of the sample (Q value). Findings were considered statistically significant at probability levels of $P < 0.05$. All statistical analysis was performed using JMP Release 5.1.1 software (SAS Institute Inc, Cary, NC, USA) .

Results

The clinical and laboratory data are summarized in Table 1. The cohort observed in the present study was similar to other large series of patients (Service *et al.* 1991) with hypoglycaemia syndrome having a slight female predominance (53%), median of age in the fifth decade (43 year-old), an elevated insulin level and a markedly decreased fasting glucose level. Disease duration ranged heterogeneously from 1 to 168 months, median of 24 months. The largest diameter of the tumours ranged from 6 to 55 mm (median 16 mm). Nuclear atypia was considered as mild in 55% (10/18) and moderate in 45% (8/18) of tumour samples. Mitotic index ranged from 0 to 4 mitosis/HPF. There was no evidence of perineural invasion and vascular in any samples. Immunoperoxidase staining for chromogranin A and synaptophysin were positive in all tumour tissues. Insulin was present with strong, moderate and weak immunostaining in 44%, 28%, and 22% respectively. Although metastatic tumour tissue from patient #16 did not present immunohistochemistry for insulin, this patient had unequivocal hypoglycaemia syndrome. Three tumour tissues had weak immunoreaction for somatostatin (patients #10, #11 and #15), one had strong

Figure 1 Representative photomicroscopic images of immunohistochemical analyses in insulinomas: (A) Immunoreactive cells for insulin showing a rather wide distribution ($\sim \times 20$); (B) Moderate immunostaining for glucagon ($\sim \times 20$); (C) somatostatin immunostaining shows only a few collection of cells ($\sim \times 20$); and (D) Ki-67 proliferative index with $>2\%$ of positive cells ($\sim \times 40$).

immunostaining for glucagon (primary tumour from patient #16) and none for gastrin. Positive immunoreactivity for Ki-67 in $>2\%$ cells was demonstrated in 22% of all studied samples. Tumour protein p53 was not detected by immunohistochemistry in any tumour, including the two hepatic metastases. Figure 1 shows representative immunohistochemical images of insulin (A), glucagon (B), somatostatin (C), and Ki-67 (D). All relevant histopathological and immunohistochemical features are depicted in Table 2.

Semi-quantitative RT-PCR of SSTR subtypes mRNA revealed that SSTR subtypes 1, 3, and 5 were expressed in 100%, SSTR2 in 89%, and SSTR4 only in 22% of the insulinomas. SSTR5 mRNA expression (by semi-quantitative RT-PCR) was significantly higher in tumours with histopathological features related to an aggressive behaviour. These findings led us to confirm the SSTR5 mRNA expression by

real-time quantitative PCR (Figure 2) in all tumoural samples. SSTR5 mRNA expression levels in insulinomas from patients, classified accordingly to WHO criteria are depicted in Figure 3 which shows a statistically significant increase in SSTR5 mRNA expression (expressed as Q values) in WDET-UB [median (min-max); 0.75 (0.53–0.97)] in comparison to WDET-BB [0.31 (0.03–0.69)], $P < 0.05$. The primary carcinoma (WDEC) and the metastases presented SSTR5 mRNA levels as high as the WDET-UB.

Figure 4 depicts SSTR5 mRNA expression levels insulinoma samples classified accordingly to their size (upper panel) nuclear atypia (lower panel). SSTR5 mRNA expression was significantly higher in insulinomas larger than 15 mm (<15 mm: 0.28 (0.03–0.47) vs ≥ 15 mm: 0.75 (0.53–0.97)] and moderate nuclear atypia [mild: 0.39 (0.03–0.75) vs. moderate: 0.81 (0.25–0.97)], $P < 0.05$.

Table 2 Histopathological and immunohistochemical findings of insulinomas from the patients studied. Magnitude of immunohistochemical expressions positivity was semi-quantitatively represented by number of crosses

Patient	Histopathological analysis			Immunohistochemical analysis				Histological classification
	Tumor size (mm)	Nuclear atypia	Mitotic index (/10HPF)	Insulin	Glucagon	Somatostatin	Ki-67 (% cells)	
1	6	Mild	1	+++	—	—	<2	WDET-BB
2	8	Mild	0	+++	—	—	<2	WDET-BB
3	10	Moderate	1	+++	—	—	<2	WDET-BB
4	17	Mild	0	+++	—	—	<2	WDET-BB
5	15	Mild	0	++	—	—	<2	WDET-BB
6	10	Mild	0	+++	—	—	<2	WDET-BB
7	14	Mild	0	+++	—	—	<2	WDET-BB
8	13	Mild	0	+++	—	—	<2	WDET-BB
9	7	Mild	1	+	—	—	<2	WDET-BB
10	15	Moderate	2	++	—	+	<2	WDET-UB
11	55	Moderate	2	+++	—	+	<2	WDET-UB
12	20	Moderate	0	++	—	—	>2	WDET-UB
13	20	Mild	0	++	—	—	<2	WDET-UB
14	20	Moderate	1	+	—	—	<2	WDET-UB
15	15	Moderate	1	++	—	+	>2	WDET-UB
16	20	Moderate	1	+	+++	—	>2	WDEC
17	10	Mild	3	—	—	—	>2	MEC
17	15	Moderate	2	+	—	—	<2	MEC

HPF: high-power field; Ki-67: proliferation-related Ki-67 antigen; WDET-BB: well-differentiated endocrine tumour with benign behaviour; WDET-UB: well-differentiated endocrine tumour with uncertain behaviour; WDEC: well-differentiated endocrine carcinoma; MEC: metastasis of endocrine carcinoma.

SSTR5 mRNA expression and tumour size presented a statistically significant positive correlation ($\rho = 0.71$, $P < 0.05$) as shown in Figure 5.

As shown in Figure 6, SSTR5 mRNA expression in primary insulinomas [0.36 (0.03–0.75)] was significantly lower than in primary plurihormonal tumours [0.83 (0.53–0.97)] either WDEC-BB or WDETC-UB, $P < 0.05$.

Noteworthy, the arrows in figures 3–6 indicate a 64 year-old male (patient #14) with a short history of classic Whipple’s triad of hypoglycaemic symptoms for a month. Histopathological examination revealed a tumour of 20mm resected from the neck of the pancreas exhibiting moderate nuclear atypia and mitotic index of 1/10 HPF (Table 2). Histopathological classification referred this insulinoma as well-differentiated endocrine tumour with uncertain behaviour. A sample of this tissue was disrupted, trypsinised and cultured cells were able to grow in soft agar medium. Although this growth pattern suggests a malignant phenotype, tumour-associated antigen (Ki-67) was positive in <2% of the cells (Table 2) and SSTR5 mRNA expression was low, features more compatible with a less aggressive biological behaviour. For these heterogenous characteristics,

this tumour was excluded from the all analyses performed.

Discussion

Analysis of SSTR subtypes mRNA expression in a series of 18 insulinomas by semi-quantitative RT-PCR showed that SSTR subtypes 1, 3 and 5 were expressed in the totality of the studied insulinomas and SSTR subtype 2 was expressed in all but 2 tumours (89%), while SSTR subtype 4 was expressed in only four insulinomas (22%). SSTR distribution observed in this series does not agree entirely with the distribution previously reported which also employed RT-PCR analysis (Kubota *et al.* 1994, Jais *et al.* 1997, Papotti *et al.* 2002, Bertherat *et al.* 2003). However, frequencies of expression of SSTR subtypes 1, 5 and 4 were similar to at least one previous report. Briefly, SSTR subtype 1 and SSTR5 frequencies of 100% were reported in two small series (Kubota *et al.* 1994, Jais *et al.* 1997, respectively) and SSTR subtype 4 frequency of 20% was observed by Bertherat *et al.* (2003). The frequency of SSTR subtype 3 expression (100%) observed in this series was higher than the maximum reported in other series (84%) (Papotti *et al.* 2002).

Figure 2 SSTR5 mRNA expression analysis in insulinoma samples from patients #2 and #8. Real-time qPCR amplification curves for comparing SSTR5 and PSMC6 mRNA expressions in duplicates. Upper panel shows electrophoresis on 3% agarose gel of amplified products of the same samples graphically depicted below.

The SSTR subtypes sequence homologies could be account for these conflicting results since different primer sets might be employed in evaluating for SSTR subtypes mRNA expression. We have managed to pick up primers set, paired to the most non-homologous regions sites as possible of SSTR isoforms, in order to circumvent any misinterpretation of the data.

An antioncogenic role has been attributed to SSTR subtype 2 in human pancreatic cancer (Delesque *et al.* 1997, Rochaix *et al.* 1999) and high levels of SSTR subtype 2 expression were positively related to a favorable prognostic in neuroblastomas (Sestini *et al.* 1996), breast cancers and, maybe, colorectal cancer (Buscail *et al.* 1996, Raggi *et al.* 2002). Thus, even knowing the benign nature of most insulinomas, we evaluated whether the expression of SSTR subtypes may correlate with specific histopathological data and cell proliferation markers. There was no association between lower SSTR subtype 2 mRNA expression and variables suggestive of a more aggressive biological behaviour. However, a positive relationship was observed between SSTR5 mRNA expression and histopathological features related to tumour aggressiveness, as expressed either by the criteria considered by WHO International Classification of Endocrine Tumours or by larger tumour sizes and higher number of cells with nuclear atypia. Furthermore, a positive correlation between tumour size and SSTR5 mRNA expression was also detected.

Since there is evidence supporting the involvement of SSTR5 in SST-induced anti-proliferative effects (Cordelier *et al.* 1997, Sharma *et al.* 1999), an

Figure 3 SSTR5 quantitative mRNA expression in insulinoma samples from patients classified accordingly to WHO International Histological Classification of Endocrine Tumours, i.e., WDET-BB, well-differentiated endocrine tumour with benign behaviour; WDET-UB, well-differentiated endocrine tumour with uncertain behaviour; WDEC, well-differentiated endocrine carcinoma; and MEC, metastasis of endocrine carcinoma. Q values were calculated as described in the statistical analysis section. The ends of the box are the 25th and 75th quantiles, respectively. The difference between the quartiles is the interquartile range. The line across the middle of the box identifies the median sample value. Each box has lines extending from the ends of the box to the outermost data point that falls within the distances computed. Arrow is indicating patient #14 as mentioned in the text.

association between SSTR5 mRNA expression and features indicative of increased cell proliferation was unexpected. A discrepancy between SSTR5 mRNA and protein levels may not be ruled out since immunohistochemistry study was not performed. However, previous immunohistochemistry studies have shown the presence of SSTR5 receptors in insulinomas (Papotti *et al.* 2002, Kulaksiz *et al.* 2002, Patel *et al.* 2002) and Bertherat *et al.* (2003) demonstrated significant binding of a SSTR5 ligand in a series of 20 insulinomas, which argues in favour of the presence of functional SSTR5 receptors in insulinomas. Furthermore, the positive association between SSTR5 mRNA expression and at least two variables related to cell proliferation and tumour aggressiveness support a biological relevance for these findings. Considering the inhibitory effects of activated SSTR5 on the cell cycle, it is possible that SSTR5 augmented expression constitutes a cellular response to increased proliferation, a compensatory mechanism that tries to control aberrant cell proliferation.

The positive correlation between SSTR5 expression and tumour size suggests that studying the effect of SSTR5 analogues in insulinomas may be an interesting

Figure 4 SSTR5 quantitative mRNA expression in insulinoma samples from patients classified accordingly to tumour size (upper panel) and nuclear atypia (lower panel). Arrows on both panels are indicating patient #14 as mentioned in the text. Please, see legend of figure 3 for more detailed explanations. (○, WDET-BB; □, WDET-UB; ●, WDEC; ■, MEC; and ◻, tumour with grow in soft agar medium, patient #14).

approach. Octreotide and lanreotide bind preferentially to SSTR subtype 2 and, in our experience, as much as in several reports in the literature (Dogliotti *et al.* 2001), limited clinical responses concerning control of both insulin secretion and tumoural growth have been observed following their use in the treatment of insulinomas. SOM-230 is a novel SST analog that, compared with octreotide, demonstrates a 30- to 40-fold higher affinity for SSTR subtypes 1 and 5 (Bruns *et al.* 2002). In the present study, these two SSTR subtypes were present in 100% of the tumours. Given that SSTR1 and SSTR5 may have an inhibitory effect on cell proliferation and that SSTR5 is involved in

Figure 5 Correlation analysis between SSTR5 quantitative mRNA expression and insulinoma size showing a statistically significant positive Spearman's coefficient (ρ) of correlation. Please, see legend of figure 3 for more detailed explanations. (○, WDET-BB; □, WDET-UB; ●, WDEC; ■, MEC; and ◻, tumour with grow in soft agar medium, patient #14).

Figure 6 SSTR5 quantitative mRNA expression in insulinoma samples from patients classified accordingly to their hormonal functionality: primary tumours (PT), plurihormonal primary tumours (PP) and metastases (M). Please, see legend of figure 3 for more detailed explanations. (○, WDET-BB; □, WDET-UB; ●, WDEC; ■, MEC; and ◻, tumour with grow in soft agar medium, patient #14).

insulin secretion control (Lamberts *et al.* 2002), SOM-230 would have the potential to promote size reduction and inhibition of insulin secretion in insulinomas.

SSTR5 mRNA expression was significantly higher in primary plurihormonal tumours which expressed SST and glucagon in comparison to primary insulinomas that did not express these hormones. Previous *in vitro* studies suggest that SST upregulates its receptors. An exposure of SST for 48 hours significantly increases receptor binding and SSTR subtypes 1–5 mRNA levels in a pituitary cell line (Bruno *et al.* 1994). Agonist

treatment of stable CHO-K1 cells individually expressing the five human SST subtypes elicit up-regulation of SST subtype 2 and 5, after 60 min exposure (Hukovic *et al.* 1996). Although prolonged exposure to SST has not been evaluated, these results suggest a modulation of SST expression by SST, but further *in vitro* studies are necessary to address this issue in pancreatic β cells. Moreover, it would be reasonable to accept that expression of more than one hormone in insulinoma, especially glucagon, would be related to a more aggressive behaviour since glucagonomas are rare gastrointestinal tumours considered to be malignant in the 200 cases reported so far (Soga & Yakowa 1998). It is possible that plurihormonal insulinomas present a more aggressive behaviour in comparison to pure insulinomas.

In conclusion, this is the first study that demonstrates an association between the expression of a SST subtype and variables related to the biological behaviour in insulinomas. The positive correlation between SST5 expression and tumour size suggests that the use of SST analogues more specific to SST5 in the treatment of insulinomas deserves attention.

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