Gastrointestinal stromal tumors express the orexigen ghrelin

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Abstract

Expression of the neuroendocrine marker synaptic vesicle protein 2 (SV2) has been reported in a few cases of gastrointestinal stromal tumors (GISTs). The goal of the present study was to assess the relevance of this finding and identify a possible hormone production in these tumors. We chose to study the orexigen ghrelin and its receptor, since these patients are seldom cachexic, even in advanced disease stages. We investigated ghrelin expression by means of immunohistochemistry on frozen or paraffin-embedded sections from 22 GISTs from a well-characterized patient material. Expression of the growth hormone secretagogue receptor, the ghrelin receptor, was investigated in a subset of lesions. In six tumors, mRNA levels of ghrelin, the ghrelin receptor, and SV2 were analyzed by real-time quantitative PCR. Totally 17 out of 22 tumors showed immunoreactivity for ghrelin. Five out of ten tumors were immunoreactive for the ghrelin receptor, and all of these co-expressed ghrelin. All tumors expressed ghrelin, ghrelin receptor, and SV2 mRNA. GISTs frequently express SV2, ghrelin, and its receptor, indicating the presence of autocrine/paracrine loops.

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Introduction

Gastrointestinal stromal tumors (GISTs) originate from pacemaker cells (the interstitial cells of Cajal) and therefore exhibit a phenotype with expression of CD34 and the KIT tyrosine kinase receptor (Kindblom *et al.* 1998, Sarlomo-Rikala *et al.* 1998, Corless *et al.* 2004). Activating *KIT* mutations are seen in a majority of GISTs (Hirota *et al.* 1998), most commonly in exon 9 or 11. Subsets of GISTs can have mutations in the *KIT*-related kinase gene *PDGFRA* (Corless *et al.* 2004).

Patients with neurofibromatosis-1 (NF-1) have an increased risk of developing GIST, and in a population-based consecutive series of 259 patients with GIST (1983–2000), four patients had NF-1 and five other patients harbored concomitant neuroendocrine tumors (Nilsson *et al.* 2005). Mutations of *NF-2* have been reported in single cases of GIST (Fukasawa *et al.* 2000). GIST can also occur with paraganglioma and pulmonary chondroma in the setting of Carney triad (Carney *et al.* 1977, Bummig *et al.* 2006).

Clinically, GISTs range from small, indolent, surgically curable tumors to very aggressive tumor disease. Histopathologically, they are composed of spindle or epithelioid cells, or are of a mixed type. According to a recently suggested classification, the risk of malignancy can be estimated based on tumor size and mitotic count (Fletcher *et al.* 2002). Until recently, surgery was the only effective treatment, with poor outcome for high-risk patients (DeMatteo *et al.* 2000). With the introduction of selective tyrosine kinase inhibitors, the medical treatment of patients with advanced GIST harboring *KIT* or *PDGFRA* mutations has markedly improved (Bummig *et al.* 2003, Corless *et al.* 2004).

Patients with GIST are, unlike other patients with malignant abdominal tumors, seldom cachexic, even in advanced stages of disease (personal experience). A similar relative well-being can also be seen in patients with endocrine pancreatic tumors (EPT), which recently were shown to express ghrelin, a potent

orexigenic hormone (Iwakura *et al.* 2002, Volante *et al.* 2002, Corbetta *et al.* 2003, Rindi *et al.* 2004).

Ghrelin, a 28-amino acid peptide discovered in 1999, is expressed mainly in the gastric endocrine A cells, and also in other tissues, e.g., intestine, pancreas, kidney, liver, and hypothalamus (De Ambrogi *et al.* 2003). Ghrelin is a natural ligand of the growth hormone secretagogue receptor (GHS-R) and stimulates growth hormone release. It increases appetite and reduces fat utilization when given systemically or into the brain of rodents (Tschop *et al.* 2000). Levels are high in fasting individuals and fall upon intake of food. Obese individuals have lower circulating ghrelin levels compared to lean. Expression of ghrelin and its receptor have previously been shown in testicular, breast, and prostate cancer, as well as in pancreatic and other neuroendocrine tumors. Accordingly, an autocrine/paracrine role of ghrelin has been suggested (Jeffery *et al.* 2003). A ghrelinoma syndrome has not yet been characterized.

Interstitial cells of Cajal have been reported to express cholecystokinin and somatostatin receptor subtypes (Sternini *et al.* 1997, Patterson *et al.* 2001). Recently, GISTs were shown to express high numbers of other peptide hormone receptors, e.g., bombesin

subtype 2 and vasoactive intestinal peptide subtype 2 receptors (Reubi *et al.* 2004). The neuroendocrine marker synaptic vesicle protein 2 (SV2) (Portela-Gomes *et al.* 2000) has been reported in eight cases of GIST (Jakobsen *et al.* 2002). Furthermore, one patient with an insulin-like growth factor-II producing GIST has been reported with symptoms of hypoglycemia (Beckers *et al.* 2003). In this paper, we share our data on expression of the orexigen ghrelin and its receptor, as well as SV2 mRNA, in GISTs.

Materials and methods

Tumor material

GISTs from 22 patients were studied. Tumors were located in the stomach (n=8), duodenum (n=3), small intestine (n=9), and rectum (n=2); were of spindled (n=10), epithelioid (n=10), or mixed type (n=2), and displayed KIT immunoreactivity. Due to limited availability, the tumor material was retrieved from both paraffin-embedded blocks (n=12) and fresh frozen surgical specimens (n=10).

All tumors were examined histopathologically regarding size, growth pattern (spindled, epithelioid, and mixed), mitotic rate (mitoses per high power field,

Table 1 Patient data and results of ghrelin and ghrelin receptor staining of GISTs

Age/sex	BMI preop.	Tumor site and size (cm)	Tumor type	Ki67%/mitotic rate	Risk score	Kit exon 11 mutation	Distant metastases at diagnosis	Ghrelin IHC	Receptor IHC
56/M	21.4	SB/20	Mixed	10/2–5	High	Yes	Liver	–	–
56/M	ND	R/4.5	Spindled	5/<2	Low	Yes	–	–	–
74/M	26.6	D/11	Epithelioid	25/6–11	High	No	–	++	++
63/M	25.3	S/16	Epithelioid	1/<2	High	Yes	Liver	–	–
57/M	27.6	SB/29	Mixed	30/>10	High	Yes	Peritoneal	+	–
15/F	22.9	S/7	Epithelioid	<5/2–5	Intermediate	No	–	++	+++
70/M	22.1	D/25	Spindled	25/6–10	High	No	Liver	++	+
48/M	30.1	R/9	Spindled	10/2–5	Intermediate	Yes	–	++	+
55/M	ND	SB/15	Epithelioid	>10/2–5	Intermediate	Yes*	Liver, peritoneal	+	–
58/M	ND	SB/4.5	Spindled	<5/<1	Low	Yes	–	++	ND
45/M	26.3	SB/5	Spindled	10/>10	High	Yes	–	+	++
72/M	ND	SB/12	Spindled	20/>10	High	Yes	Liver	++	ND
63/M	ND	SB/15	Epithelioid	50/2–5	High	No	–	–	ND
69/F	24.5	SB/11	Epithelioid	5/2–5	High	Yes	–	+++	ND
73/F	25.6	S/20	Epithelioid	50/>10	High	Yes	–	+++	ND
48/F	21.8	D/8	Spindled	50/>10	High	Yes	Liver	+++	ND
77/F	ND	S/4	Spindled	10/2–5	Intermediate	No	Liver	++	ND
27/M	31.1	S/3.5	Epithelioid	10/6–10	High	No	–	+	ND
72/M	ND	S/19	Spindled	25/>10	High	Yes	Lung, liver	+++	ND
72/F	20.8	S/18	Epithelioid	29/6–10	High	Yes	Liver	+	ND
48/M	ND	SB/29	Spindled	50/>10	High	Yes	Liver	–	ND
80/M	25.1	S/20	Epithelioid	10/6–10	High	Yes	Carcinomatosis	+++	ND

IHC, immunohistochemistry; S, stomach; D, duodenum; SB, small bowel; R, rectum; ND, not done; –, no immunoreactivity; BMI, body mass index; preop., preoperatively.
+, 1–25% of cells positive; ++, 26–75% of cells positive; +++, >75% of cells positive; *, exon 9 mutation.

Table 2 Ghrelin and ghrelin receptor immunoreactive tumors in relation to clinical and morphological features, genotype, and proliferation

Tumor material	Ghrelin positive	GHS-R ^a positive
Total (n=22)	17/22	5/10
Tumor type		
Spindled (n=10)	8/10	3/4
Epithelioid (n=10)	8/10	2/4
Mixed (n=2)	1/2	0/2
Location		
Stomach (n=8)	7/8	1/2
Duodenum (n=3)	3/3	2/2
Small bowel (n=9)	6/9	1/4
Rectum (n=2)	1/2	1/2
Size		
< 5 cm (n=8)	3/4	0/1
5–10 cm (n=4)	4/4	3/3
> 10 cm (n=14)	10/14	2/6
Mitotic index		
≤ 5 (n=10)	6/10	2/6
6–10 (n=5)	5/5	2/2
≥ 10 (n=7)	6/7	1/2
Ki67		
≤ 5 (n=5)	3/5	1/3
6–10 (n=6)	5/6	2/3
> 10 (n=11)	9/11	2/4
Risk index		
Low (n=2)	1/2	0/1
Intermediate (n=3)	3/3	2/2
High (n=17)	13/17	3/7
Exon 11 or 9 mut		
Yes (n=15)	11/15	2/7
No (n=6)	5/6	3/3

^aImmunohistochemistry for the ghrelin receptor was performed in a subset of lesions (n=10).

assessed in 50 high power fields), and Ki67 index. Furthermore, the tumors were graded as having a very low (n=0), low (n=2), intermediate (n=3), or high risk (n=17) of malignant behavior, according to Fletcher *et al.* (2002). A summary of tumor characteristics is given in Tables 1 and 2.

Three tumors had a low mitotic rate (<2), seven displayed a mitotic rate between 2 and 5, and in five tumors the number of mitoses was between 6 and 10. In the remaining seven tumors, more than ten mitoses per high power field were seen. Five tumors had a low Ki67 index (<5%), six tumors had a Ki67 index between 6 and 10%, and 11 tumors displayed more than 10% Ki67 immunoreactive cells. Fifteen out of twenty-one analyzed patients had an activating mutation of *KIT*. Median patient age at diagnosis was 60.5 years (range 15–80 years, mean 59 years). Median tumor size at diagnosis was 13.5 cm (range 3.5–29 cm, mean 13.9 cm), placing most patients in the high-risk

group. Twelve out of twenty-two patients had distant metastases at diagnosis.

Normal tissue material

Frozen consecutive sections of normal small intestine from a patient with a GIST tumor (patient no. 11) were used.

Immunohistochemistry (IHC)

Due to a limited availability of tumor tissue, either frozen (n=10) or paraffin-embedded (n=12) sections were used for ghrelin IHC. Staining for the ghrelin receptor was done only on frozen tissue, except in one case where both frozen and paraffin-embedded materials were available. Frozen sections (6 μm) were fixed in acetone, and incubated at 4 °C overnight with rabbit anti-ghrelin (H-031-30, Phoenix Pharmaceuticals, Belmont, CA, USA; 1:2400) or rabbit anti-GHS-R (H-80, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), diluted in PBS with 1% BSA. The reaction product was visualized using a biotinylated secondary rabbit antibody, VECTASTAIN *Elite* ABC (Vector, Burlingame, CA, USA) and the chromogen 3-amino-9-ethylcarbazol (Sigma). Counterstaining was done with Mayer’s hematoxylin. For antigen retrieval, the paraffin-embedded sections were subjected to pretreatment (2×5 min microwave heating at 900 W in Tris–EDTA at pH 9). Sections were incubated with rabbit anti-human ghrelin (GHS-11-A, Alpha Diagnostics, San Antonio, TX, USA; 1:50), diluted in antibody diluent (DakoCytomation, Glostrup, Denmark), or goat anti-GHS-R (F-16, Santa Cruz Biotechnology; 1:200), diluted in PBS with 1% BSA, at room temperature for 2 h. In our hands, these antibodies generated better IHC staining on paraffin-embedded tissue compared to the antibodies used on frozen tissue. The reaction product was revealed using the Envision+ System with 3,3’-diamino-benzidine (DAB) as chromogen (DakoCytomation). Sections were counterstained with Mayer’s hematoxylin. All sections had been stained with anti-Ki67 (M7240, DakoCytomation; 1:100) and anti-KIT (sc-168, Santa Cruz Biotechnology; 1:100). Initial experiments with each antibody were performed with or without the inclusion of primary antibody. A blocking peptide (GHS-11-P, Alpha Diagnostics), 5 μg peptide/1 μg antibody, successfully demonstrated the specificity of ghrelin antibody (GHS-11-A) used on paraffin-embedded sections (Fig. 1a). All sections were examined by one pathologist and graded as being negative, or having 1–25% (+), 26–75% (++), or more than 75% positive cells (+++).

Figure 1 Light microscopy of a tumor (no. 6) showing immunoreactivity for ghrelin (paraffin-embedded tissue, $\times 200$) (a). The reactivity was abolished by preabsorbing ghrelin anti-serum with an excess of ghrelin peptide (insert). The same tumor was also immunoreactive for the GHS-R (paraffin-embedded tissue, $\times 400$) (b). Light microscopy of a tumor (no. 10) displaying strong immunoreactivity for ghrelin (frozen tissue, $\times 400$) (c).

Real-time quantitative PCR (qPCR)

Total RNA from frozen tumors was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Consecutive slides were stained with Mayer's hematoxylin and studied with light microscopy to ensure adequate tumor sampling and avoid inclusion of normal tissue.

cDNA was synthesized using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression of ghrelin (primer/probe Hs00175082_m1; Applied Biosystems) in six GIST tumors was determined by real-time qPCR, and compared with that of controls (five EPT: one IHC negative, one with single dispersed IHC positive cells, and three IHC positive tumors). Relative mRNA expression of SV2 (primer/probe Hs00372069_m1; Applied Biosystems) was determined in six GIST tumors and compared with that of four EPTs. Relative mRNA expression of the ghrelin receptor (primer/probe Hs0026978_s1; Applied Biosystems) was determined in six GIST tumors, and compared with that of four EPTs (three IHC positive and one negative). Commercially available primer and probe sets spanning exon/exon boundaries were used and measured against

standard curves generated from dilution series of human pooled cDNA or human fetal brain cDNA from Human Total RNA Master Panel II (636643; BD Biosciences, San Jose, CA, USA). Reactions were performed and analyzed using an Applied Biosystems PRISM 7700 Sequence Detector. Standard cycling conditions were used (Heid *et al.* 1996). The gene-specific signals were normalized to that of the 18S or HPRT housekeeping genes to correct for differences in RNA quantity. All TaqMan assay reagents were obtained from Applied Biosystems.

Mutation analysis

Genomic DNA was prepared from paraffin-embedded sections for nucleotide analysis of exons 9 and 11 of *KIT*. Exon 11 of *KIT* was amplified by PCR and directly sequenced and cloned, if needed. In summary, nucleotide sequence analyses were made as previously described (Bumming *et al.* 2003).

Statistical analysis

The χ^2 and one-way ANOVA tests were used; $P < 0.05$ was considered significant.

Body mass index (BMI)

The BMI was calculated as kg/m^2 when this information was available in the medical records.

Ethical approval

Permission for this study was obtained from the Ethical Committee, University of Göteborg, Sweden.

Results

IHC

Immunoreactivity for ghrelin was detected in 17 out of 22 tumors (Tables 1 and 2; Fig. 1a and c). The reaction product was confined to the cytoplasm and revealed a fine granular pattern. In several tumor samples, the staining was heterogeneous with large areas devoid of ghrelin immunoreactivity and other parts with strong staining. Five out of 17 tumors showed less than 25% ghrelin-immunoreactive cells. Seven were graded as ++ (26–75% immunoreactive tumor cells), and the remaining five tumors were graded as +++ (more than 75% immunoreactive tumor cells). Ghrelin immunoreactivity was equally common in spindled, epithelioid, and mixed phenotypes of 8/10, 8/10, and 1/2 respectively. All three duodenal tumors expressed ghrelin immunoreactivity, as did seven out of eight tumors of gastric origin. Six out of nine tumors from the small intestine and one out of two rectal tumors displayed ghrelin immunoreactivity. No correlation was seen between tumor location ($P=0.426$), size ($P=0.590$), *KIT* genotype ($P=0.935$), and ghrelin expression. Tumors with a mitotic rate of ≤ 5 expressed ghrelin in six out of ten cases versus all five lesions with a mitotic rate of 6–10, and six out of seven with a mitotic rate exceeding 10. However, a mitotic count >5 did not correlate significantly with the presence of ghrelin immunoreactivity ($P=0.210$). Neither did Ki67 index >5 ($P=0.659$). All three tumors classified to be of intermediate risk, and 13 out of 17 high-risk tumors, showed ghrelin immunoreactivity. On the other hand, one out of two low-risk tumors were also immunopositive. Thus, ghrelin immunoreactivity did not show significant correlation with the risk score used ($P=0.420$).

IHC for the ghrelin receptor was performed on frozen tissue from ten surgical specimens. No correlation was seen between receptor expression and risk score ($P=0.208$), Ki67 index ($P=0.717$), mitotic rate ($P=0.264$), anatomic site ($P=0.392$), size ($P=1.000$), or morphological type ($P=0.223$). The receptor stained positive in five out of ten GISTs

(Tables 1 and 2; Fig. 1b) and all five receptor-positive tumors showed concomitant ghrelin expression. Two receptor-negative tumors were ghrelin-positive, and three were negative for both the receptor and the ligand.

A thin layer of cells in normal small bowel tissue, situated between the outer and inner intestinal muscle layers, possibly representing interstitial cells of Cajal, showed KIT immunoreactivity. These cells were negative for both ghrelin and its receptor (data not shown).

Real-time qPCR

Real-time qPCR revealed ghrelin mRNA expression in all six analyzed tumors (Fig. 2a). The mean ghrelin mRNA level in GISTs was similar to that of five EPTs used as controls (one IHC-negative, one with single dispersed IHC-positive cells, and three IHC-positive tumors). Ghrelin receptor mRNA expression was found in all six analyzed GIST lesions, with one tumor expressing much higher levels than the others (Fig. 2b). The mean receptor mRNA level in GISTs was 3.2 times higher (range 0.69–13.6, median 1.1) than controls, which did not constitute a significant difference ($P=0.420$). RNA levels of ghrelin and its receptor did not correlate to the degree of protein expression detected by IHC. High levels of SV2 mRNA expression were found in all six analyzed tumors (Fig. 3). SV2 mRNA levels in GIST tumors were similar to those of the EPTs used as controls (mean 1.4 times higher, range 0.64–2.0, median 1.6).

BMI

Data for calculation of BMI were available in 14 patients (Table 1). Mean BMI preoperatively was 25.1 ± 3.1 (s.d.) (range 20.8–31.1, median 25.2), i.e. this patient group was slightly overweight according to the international definitions. The corresponding figure for patients with ghrelin-immunoreactive tumors was 25.4 (range 20.8–31.1, median 25.4). More than half of the analyzed GIST patients were overweight (BMI > 25), and two patients (14%) were obese (BMI > 30) (both with ghrelin-immunoreactive tumors). No patient was underweight (BMI < 20).

Discussion

The present paper is, to our knowledge, the first report of ghrelin production in GISTs. We demonstrate expression of ghrelin and its receptor mRNA in all GISTs analyzed, as well as ghrelin and receptor immunoreactivity in a majority of tumors. We also show high levels of SV2 mRNA, an established marker

Figure 2 Mean relative gene expression of ghrelin (a) and ghrelin receptor (b) determined with real-time qPCR, $\pm$ s.e.m. The mean ghrelin mRNA level in GISTs was similar to that of five pancreatic endocrine tumors (EPT 1–5) used as controls. The mean receptor mRNA level in GISTs was 3.2 times higher (range 0.69–13.6) versus four EPT (EPT 1–4).

for neuroendocrine cells, in all tumor samples. Levels were comparable with those of EPT, a tumor group known to have a strong expression of SV2 protein.

Previous reports have demonstrated expression of peptide hormone receptors on both interstitial cells of Cajal and GISTs. The presence of immunoreactivity for both ghrelin and its receptor indicates a possible autocrine/paracrine loop. Co-expression of the hormone and its receptor has been shown in several tumor types, including pancreatic adenocarcinoma and EPT (Jeffery *et al.* 2003). Ghrelin has been shown to increase proliferation and invasiveness in a pancreatic adenocarcinoma cell line expressing the ghrelin receptor (Duxbury *et al.* 2003). In the present study

of a limited patient material, we did not find any correlation between the expression of ghrelin and its receptor and malignant features (e.g. tumor size, high-risk score, *KIT* exon 11 mutations, or disseminated disease). Larger numbers of GISTs may be necessary to address this important question.

We found ghrelin immunoreactivity in the cytoplasm with a fine granular pattern. In several tumor samples, the staining was heterogeneous with large areas devoid of ghrelin immunoreactivity and other parts with strong staining. The highly sensitive qPCR method revealed ghrelin mRNA in all examined GISTs, whereas IHC showed ghrelin reactivity in 77%. The PCR revealed mean ghrelin levels clearly above those of

Figure 3 Mean relative gene expression of SV2 determined with real-time qPCR, $\pm$ S.E.M. High levels were found in all six analyzed lesions. The mean SV2 mRNA level in GIST tumors was similar to that of EPTs 1–4 used as controls.

one immunohistochemically negative EPT and one with few ghrelin reactive cells. Two GISTs had levels similar to controls. The discrepancy between RNA levels and protein expression might be due to the observed heterogeneous distribution of ghrelin within each single sample. It could also be due to a variable degree of translation of the protein. An alternative interpretation could be that the protein is produced, but not stored, in individual tumors.

A similar expression pattern was seen for the ghrelin receptor; mRNA was revealed in all analyzed tumors, but only 50% showed immunoreactivity. One tumor had mRNA levels much higher than any of the EPT used as controls; all tumors expressed receptor mRNA at levels comparable to or even higher than the mean levels of the EPT.

Patients with GIST seldom suffer from cancer cachexia, even in advanced disease. In the present limited series of GIST patients, we could record a mean BMI of 25.1 (25.4 in patients with ghrelin-immunoreactive tumors), which shows that these patients did not suffer from cancer cachexia, despite a majority of patients presenting with large tumors (>10 cm; 8/14), high Fletcher risk score (12/14), and distant metastases (7/14). A more comprehensive patient material would be needed to statistically correlate ghrelin immunoreactivity to BMI. The BMI of our patients in the present paper indicates that GIST patients follow the trend of prevalent overweight presently seen in Sweden (Sundquist *et al.* 2004). Endogenous secretion of the potent orexigen ghrelin from the tumor might contribute to the relative well-being of these patients.

However, the analysis of circulating ghrelin levels is lacking in the present retrospective study. A more comprehensive metabolic evaluation of GIST patients in a prospective study design would be of great interest.

Conclusion

GISTs frequently express SV2, as well as the orexigenic hormone ghrelin and its receptor, indicating the presence of an autocrine/paracrine loop.

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