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Ghrelin and Obestatin in Human Neuroendocrine Tumors: Expression and Effect on Obestatin Levels after Food Intake

Malin Grönberg[a] Apostolos V. Tsolakis[a] Ulf Holmbäck[b] Mats Stridsberg[c]
Lars Grimelius[d] Eva T. Janson[a]

[a]Section of Endocrine Oncology, Department of Medical Sciences, [b]Department of Public Health and Caring Sciences, Clinical Nutrition and Metabolism, [c]Section of Biochemical Endocrinology, Department of Medical Sciences, and [d]Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden

Key Words
Obestatin · Ghrelin · Neuroendocrine tumors · Immunohistochemistry

Abstract
Background: Ghrelin and obestatin are derived from the same peptide hormone precursor and are mainly produced by the gastric mucosa. Ghrelin is involved in many biological processes, whereas the physiological function of obestatin needs further investigation. The aims of the present study were to establish the incidence of ghrelin- and obestatin-immunoreactive cells in a comprehensive panel of human neuroendocrine tumors (NETs) and to investigate if blood obestatin concentrations are influenced during a standardized meal stimulation test in healthy individuals and patients with NETs. ***Materials and Methods:*** The expression of ghrelin and obestatin was investigated in NETs (n = 149) and other endocrine-related disorders (n = 3) using immunohistochemistry with specific polyclonal antibodies. Coexpression of the peptides was evaluated by double immunofluorescence. Concentrations of obestatin in blood were measured during a meal test in 6 healthy individuals and 5 patients with pancreatic NETs. ***Results:*** Ghrelin and obestatin were expressed in 14/152 and 19/152 tumor tissues, respectively, mainly representing NETs of foregut origin and in pancreatic tissue from a nesidioblastosis patient. Double immunofluorescence staining showed colocalization of the peptides. During the meal test, obestatin levels in blood were unchanged in all patients but decreased significantly in the healthy individuals. ***Conclusion:*** Only a minority of NETs express ghrelin and obestatin. However, analysis of patients with tumors originating from tissues that express the peptides in normal conditions could be of importance. The results from the meal test indicate that the hormone levels are affected by food intake in healthy individuals, whereas obestatin levels remained unchanged in pancreatic NET patients.

Introduction

Ghrelin was first identified as the natural ligand for the growth hormone secretagogue receptor (GHSR) 1a. This hormone is generated by processing of a 117-amino acid peptide, termed preproghrelin. Ghrelin can be further processed by addition of an octanoyl group to the serine-3 residue, which is important for the biological activity of the peptide. Ghrelin is a multifunctional peptide and besides mediating growth hormone release through GHSR, it exerts various endocrine and non-endocrine functions [1–5].

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E-Mail karger@karger.com
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Malin Grönberg
Department of Medical Sciences, Section of Endocrine Oncology
Uppsala University
SE–751 85 Uppsala (Sweden)
E-Mail malin.gronberg@medsci.uu.se

Table 1. Obestatin and ghrelin expression in various NETs and endocrine-related disorders

Tumor type	Obestatin		Ghrelin	
	number of IR cases	relative incidence of IR tumor cells	number of IR cases	relative incidence of IR tumor cells
Medullary thyroid cancer	1/7	occas.	1/7	occas.
Primary	1/6		1/6	
Metastatic	0/1		0/1	
Parathyroid adenoma	0/7		0/7	
Adrenal cortex adenoma	0/6		0/6	
Pheochromocytoma	0/4		0/4	
Paraganglioma	0/7		0/7	
Lung NET				
Typical	4/11	occas.	2/11	occas.
Atypical	0/5		0/5	
Ovarial NET	0/4		0/4	
Merkel cell cancer	0/2		0/2	
Esophageal NET	1/1	occas.	1/1	occas.
Esophageal NEC	2/3	occas.	0/3	
Duodenal NET	1/13	occas.	1/13	occas.
Ileocecal NET	0/14		0/14	
Goblet cell carcinoid	0/3		0/3	
Rectal NET	2/12	occas.	2/12	occas.
Rectal NEC	0/6		0/6	
Presacral NET	0/2		0/2	
Pancreatic NET				
Sporadic				
Non-functioning	0/6		0/6	
Insulinoma	2/13	occas.	2/13	occas.
Gastrinoma	1/5	occas.	0/5	
Glucagonoma	1/4	occas.	0/4	
VIPoma	0/2		0/2	
Somatostatinoma	0/1		0/1	
Serotonin-producing	1/1	occas.	1/1	occas.
ACTHoma	0/3		0/3	
Primary	0/2		0/2	
Metastatic	0/1		0/1	
PTH-RPoma	0/1		0/1	
Hereditary				
MEN1	2/5	occas.	3/5	
vHL	0/1		0/1	occas.
Endocrine-related disorders				
Nesidioblastosis	1/1	occas.	1/1	occas.
C-cell hyperplasia	0/2		0/2	
Total	19/152		14/152	

All cases were primary tumors unless otherwise indicated.

ACTH = Adrenocorticotropic hormone; NEC = neuroendocrine carcinoma; PTH-RP = parathyroid hormone-related peptide; vHL = von Hippel-Lindau; VIP = vasoactive intestinal peptide. Occasional = <5%; occas. = occasional.

In 2005, another 23-amino acid peptide, obestatin, which derives from the processing of preproghrelin, was discovered. Initially, obestatin was reported to activate the G-protein-coupled receptor GPR39 and to regulate food intake and reduce gastric emptying [6]. These actions have since then been questioned [7].

The distribution of ghrelin and obestatin has been demonstrated in various tissues, where their coexpression has been described [8–12]. In a panel of neuroendocrine tumors (NETs), obestatin was expressed in a small fraction of thyroid, parathyroid, gastrointestinal and pancreatic tumors, and in most cases it colocalized with ghrelin [13]. Other studies have also demonstrated expression of these peptides in a variety of NETs, with gastric NETs showing the highest relative incidence of tumor immunoreactive (IR) cells [10, 14–17].

Furthermore, NETs originating from the gastric mucosa, pancreas, gallbladder and rectum, displaying ghrelin-IR tumor cells, have been shown to secrete ghrelin causing hyperghrelinemia [18–20]. In addition, ghrelin-IR cells have been identified in various hyperplasia patterns, in foci of neuroendocrine cell hyperplasia localized in the mucosa adjacent to gastric NETs, but in these cases, circulating ghrelin levels remained normal [17, 21, 22].

Circulating blood levels of obestatin have been measured in patients with gastric NETs expressing the peptide. A few patients had slightly elevated obestatin concentrations, but no correlations were observed with the clinicopathological data examined [10].

Our hypothesis was that obestatin should be expressed in NETs derived from the same tissues which express the peptide under normal conditions and that food intake would influence obestatin blood levels. The aim of this study was to elucidate the incidence of ghrelin- and obestatin-IR cells in a broad panel of human NETs in order to establish their role as immunohistochemical markers of such tumors. We also investigated whether obestatin blood concentrations were influenced during a meal test in healthy individuals and patients with pancreatic NETs.

Materials and Methods

Patients and Tumors

Paraffin-embedded tissue specimens were collected from the laboratory of Pathology at the University Hospital in Uppsala, Sweden. All cases (summarized in table 1) had a verified diagnosis of a NET (n = 149) or an endocrine-related disorder (n = 3) according to the WHO criteria. All cases were obtained from surgically removed material except one esophageal, one duodenal and one

rectal case that were removed by polypectomy. The esophageal tumor was located in the distal part of the esophagus.

Immunohistochemistry

The primary antibodies used for immunostaining were anti-obestatin, for which the production and characterization has been previously described [8], and anti-ghrelin (H-031-30, Phoenix Pharmaceuticals, Belmont, Calif., USA). Both antibodies were used at a dilution of 1:8,000.

The paraffin blocks were cut into approximately 4-μm sections and attached to positively charged glass slides (Superfrost Plus, Menzel Gläser, Braunschweig, Germany). The consecutive sections were immunostained using the Dako EnVision Plus-HRP Detection Kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions. For antigen retrieval, the sections were subjected to pretreatment (microwave heating for 10 min at 700 W followed by 15 min at 380 W using Tris-HCl-buffered saline, pH 8.0). The sections were incubated with the primary antibodies in PBS with 1% BSA overnight at 4°C. Bound antibodies were visualized by incubation with liquid 3, 3′-diaminobenzidine substrate chromogen for 5 min. Photographs were taken using a Zeiss Observer Z1 microscope and the Axiovision software (Carl Zeiss, Göttingen, Germany).

Immunofluorescence

Paraffin-embedded sections were deparaffinized and subjected to pretreatment for antigen retrieval (microwave heating 10 min at 700 W in Tris-HCl-buffered saline, pH 8.0). Sections were incubated 30 min with blocking solution (donkey serum, Jackson ImmunoResearch, Newmarket, UK, diluted 1:5 in PBS).

For double immunofluorescence staining, the sections were incubated for 1 h in blocking solution containing chicken anti-ghrelin (1:400, Phoenix Pharmaceuticals, Burlingame, Calif., catalogue No. Y-031-44) and rabbit anti-obestatin (1:400). Sections were washed in PBS with 0.05% Tween-20 and incubated for 1 h at room temperature with secondary antibodies: donkey anti-chicken conjugated to TRITC (1:100, Jackson ImmunoResearch) and donkey anti-rabbit conjugated to FITC (1:100, Dako Cytomation, Glostrup, Denmark) diluted in blocking solution. Appropriate washing in PBS with 0.05% Tween-20 was performed between each step, and incubation was performed in a dark moist chamber. Nuclei were counterstained with 4′, 6-diamindino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, Calif., USA). Tissues were photographed by an Axiocam HRm camera employing the Axiovision imaging software using a ×63 PlanApochromat objective and a Zeiss Axioplan 2 microscope (Carl Zeiss).

The following cases were examined with double immunofluorescence: (1) a duodenal pancreatic NET, (2) a rectal pancreatic NET and (3) a multiple endocrine neoplasia type 1 (MEN-1)-associated pancreatic NET. All the above cases were IR for both hormones in question.

Standardized Carbohydrate-Rich Meal Test

In 5 patients with pancreatic NETs and in 6 control subjects, a carbohydrate-rich meal test (560 kcal) was performed as previously described [23]. Four of the 5 patients had MEN-1. Two of the 4 cases with MEN-1 had multiple enterochromaffin-like cell carcinoids due to duodenal gastrinomas (Zollinger-Ellison syndrome), and non-functioning pancreatic NET, respectively. In the remaining 2 MEN-1 cases, one patient had a vasoactive intestinal polypeptide-producing tumor and multiple non-functioning pancreatic NETs, and the other a non-functioning pancreatic NET. The 5th case was a patient with a sporadic non-functioning pancreatic NET.

Blood Samples

Blood was collected in chilled, heparinized vacutainer tubes and centrifuged at 3,000 *g* within 30 min. Plasma was frozen in aliquots and kept at –20°C until analyzed. Obestatin concentration was measured in blood samples collected at designated time intervals (at –5, +0, +10, +20, +30, +45 and +60 min). Levels between 0.7 and 2.0 nmol/l were considered within the reference range [10].

Radioimmunoassay for Obestatin

The antibodies and the synthesized peptide were used to develop a specific RIA. For preparation of tracer, the peptide was labeled with ^{125}I (MP Biomedicals, Doornveld, Belgium) using the chloramine-T method as previously described [24]. The assay was constructed as follows: standards and unknown samples were incubated with tracer (30,000 cpm/tube) and primary antibodies at a dilution to give 30% bound radioactivity, for 3 days at 4°C. All standards and samples were assayed in duplicate. Antibody-bound radioactivity was separated from free tracer by adding a second antibody, goat anti-rabbit IgG coupled to a solid phase (SAC Cell Anti-rabbit, IDS Nordic, Herlev, Denmark). The antibody-bound radioactivity was then measured in a gamma counter (Auto gamma, Wallac, Pharmacia Biotech, Uppsala), and the data were calculated with a logit-log transformation program (Multicalc, Wallac). Plasma samples were analyzed prediluted 1:4 in assay buffer. All chemicals used were of pro analysis grade (Merck, Darmstadt, Germany). Dilutions in the RIA were performed in the assay buffer, which was a 0.05 M sodium phosphate buffer at pH 7.4, with 0.15 M sodium chloride, 0.02% sodium azide, 0.2% BSA and 0.5% Tween 20.

Statistics

The data were tested for normality with the Shapiro-Wilks test. As the data were found to be not normally distributed, the Mann-Whitney U test was used to compare controls with patients; the Wilcoxon signed rank test was used to compare baseline (time point 0) with post-breakfast challenge values (individual means of values at time point 20, 30, 45 and 60 min). Median with first and last quartiles is displayed in figure 1. For the breakfast challenge, area under the curve (AUC) was calculated using the trapezoid method. Statistical analyses were made with Statistica 10 (StatSoft, Tulsa, Okla., USA), and 0.05 was set as the significance level.

Controls

The specificity of the antibodies has been evaluated and presented previously [8]. Gastric mucosa and pancreas tissue, which were macroscopically and microscopically normal and obtained perioperatively from patients with adenocarcinoma, were used as positive controls.

Ethics

The research protocol was reviewed and approved by the local ethics committee at Uppsala University Hospital.

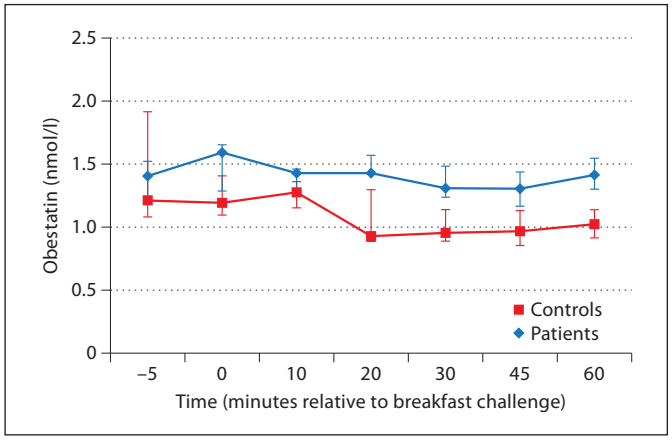

Fig. 1. Diagram showing obestatin concentrations with median and quartiles during the standardized carbohydrate-rich meal test in healthy individuals (red) and pancreatic NET patients (blue).

Results

Immunohistochemical Expression of Ghrelin- and Obestatin-IR Cells in NETs

The results are summarized in table 1. Neoplastic ghrelin- and obestatin-IR cells were found in a small fraction of NETs (fig. 2), in medullary thyroid cancer (1/7), bronchial (2/11 for ghrelin, 4/11 for obestatin), esophageal (1/4 for ghrelin, 3/4 for obestatin), duodenal (1/13), pancreatic (6/42 for ghrelin, 7/42 for obestatin) and rectal NETs (2/12). The IR cases of pancreatic NETs included insulinomas (2/13), serotonin-producing pancreatic NET (1/1), and MEN-1-associated pancreatic NETs (3/5 for ghrelin, 2/5 for obestatin). Glucagonomas (1/4) and gastrinomas (1/5) showed immunoreactivity only for obestatin. The remaining tumors were non-IR.

In the majority of IR NETs, the relative incidence of ghrelin/obestatin IR tumor cells was <5%. In total, ghrelin expression was present in 14/152 tumors, whereas obestatin could be detected in 19/152 tumors. In cases where both ghrelin and obestatin were displayed, consecutive immunostained sections indicated colocalization of the peptides.

Immunohistochemical Expression of Ghrelin- and Obestatin-IR Cells in Endocrine-Related Disorders

One case of nesidioblastosis demonstrated hyperplasia of ghrelin- and obestatin-IR cells at the periphery of the islets (fig. 2), whereas 2 cases of thyroid C-cell hyperplasia were non-IR.

Double Immunofluorescence

When both ghrelin and obestatin were expressed in the same tumor, double immunofluorescence microscopy revealed that the peptides were colocalized in the same tumor cells with identical cytoplasmic distribution (fig. 3).

Controls

In all immunohistochemical experiments, the positive control showed immunoreactivity using the antibodies in question. Scattered cells IR for ghrelin and obestatin were mainly found in the deeper third part of gastric mucosa, whereas IR cells in the pancreas were restricted to the islets and ducts.

Standardized Carbohydrate-Rich Meal Test and Radioimmunoassay Measurements

Patient and healthy subject characteristics are described in table 2. Obestatin concentrations remained within the normal reference range in all patients and healthy individuals during the standardized carbohydrate-rich meal test. Both total and post-breakfast challenge AUC for obestatin was significantly higher in patients than in the controls (see table 2). Moreover, the obestatin levels were lower in the healthy individuals after the breakfast challenge but not in tumor patients (see table 2). The concentrations with median and quartiles are given in figure 1.

Discussion

In this study, the expression of the peptide hormones ghrelin and obestatin was investigated in a large number of NETs with different embryological origin. We could demonstrate that both peptides are predominantly expressed in tumors of foregut origin but also in a few hindgut tumors. Using double-immunofluorescence stainings, colocalization of the peptides was demonstrated, which is in accordance with the results seen in normal tissue. Circulating obestatin measured during a meal test remained in the normal range for the patients with NETs. For the healthy individuals, the levels also remained in the normal range, but were lower and decreased after the breakfast challenge.

Expression of obestatin, and especially ghrelin, has previously been studied in NETs, mainly in the stomach and pancreas. Ghrelin expression in gastric endocrine tumors has been reported to be 50–80% [13, 16, 17, 21]. In pancreatic NETs, ghrelin immunoreactivity has been ob-

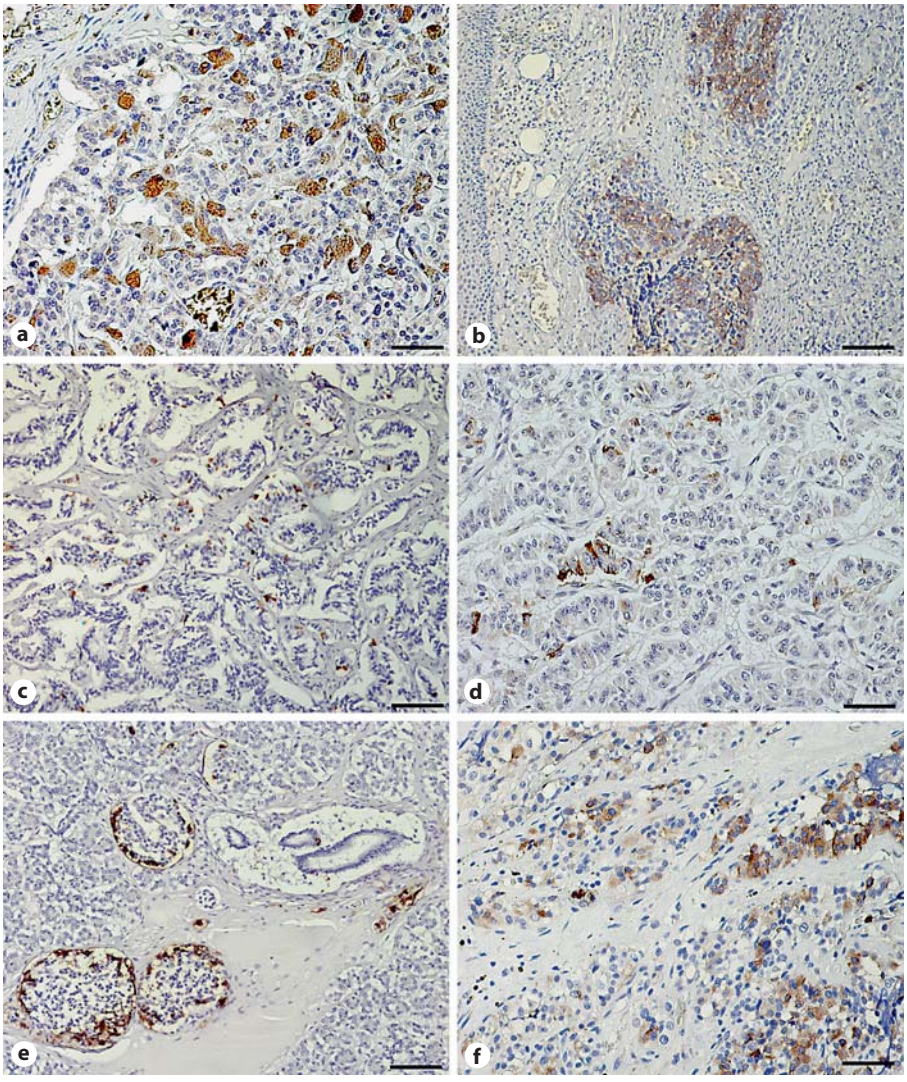

Fig. 2. NETs immunostained for obestatin. Typical bronchial (**a**), esophageal (**b**), rectal (**c**), and serotonin-producing (**d**) pancreatic NET. Only a minority of IR tumor cells are depicted. **e** Nesidioblastosis. Numerous hyperplastic islets are seen which are localized by the ducts. Hyperplasia of obestatin-IR cells is displayed in some islets. **f** Medullary thyroid cancer. **e** Single cells in the adjacent ducts are also immunostained (internal control). Scale bars: 50 μm (**a**, **d**, **f**) and 100 μm (**b**, **c**, **e**).

Table 2. Characteristics and obestatin measurement results

	Healthy individuals	Patients
Age, years	41 (32; 52)	46 (20; 63)
BMI	23.35 (22.14; 28.06)	23.58 (22.84; 42.74)
Obestatin, nmol/l	1.05 (0.92; 1.92)	1.40 (1.23; 1.65)
AUC obestatin total	68.99 (63.46;78.22)	91.76 (83.06; 97.34)*
AUC obestatin during breakfast challenge	60.04 (56.88; 68.35)	84.25 (76.82; 89.46)*
Baseline (time 0) obestatin, nmol/l	1.19 (1.10; 1.41)	1.59 (1.29; 1.65)
Post-breakfast obestatin, nmol/l	0.96 (0.87; 1.13)†	1.38 (1.31; 1.49)

Values are expressed as median (1st quartile; 4th quartile). * $p < 0.05$ vs. controls; † $p < 0.05$ vs. baseline.

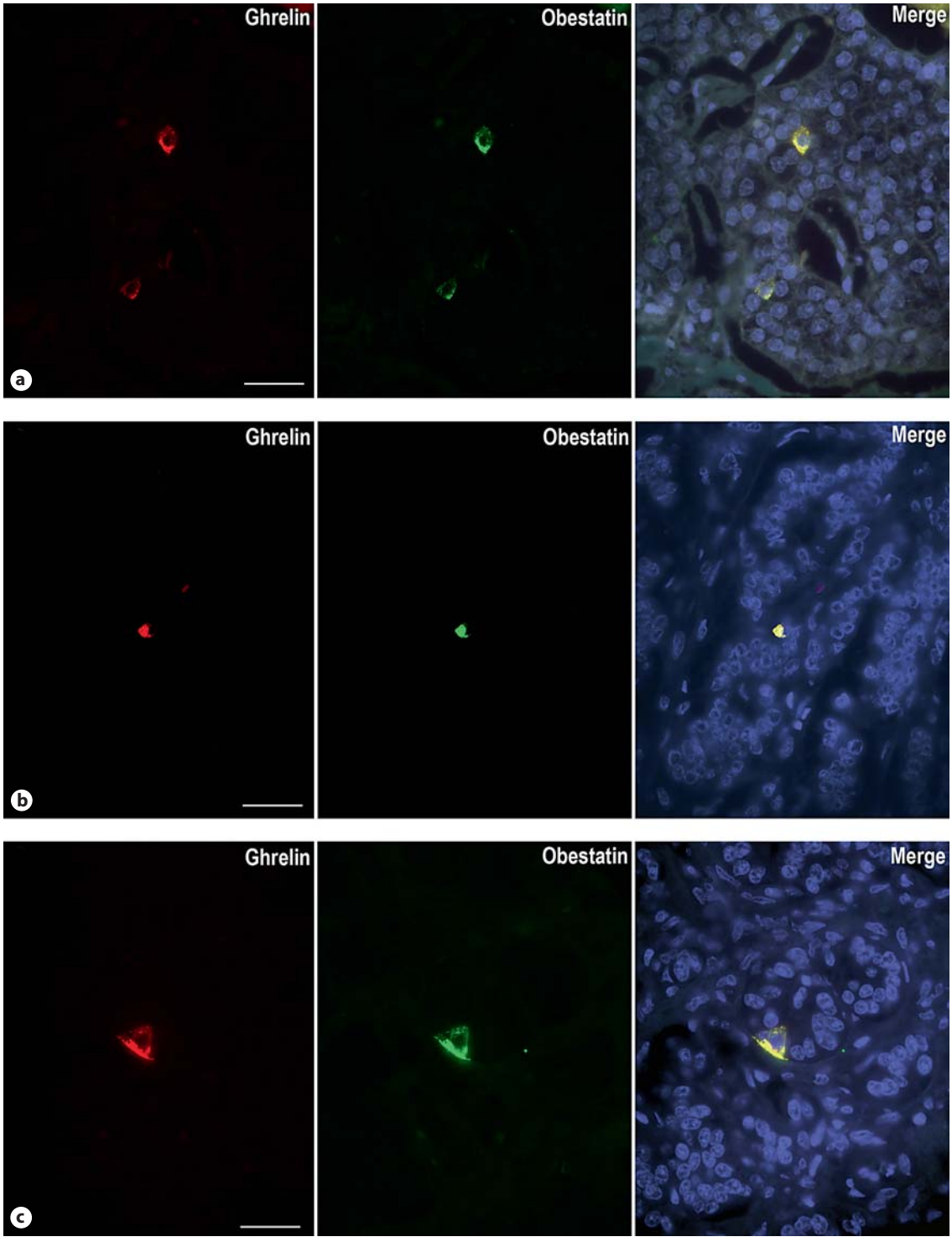

Fig. 3. Double immunofluorescence of NETs. Duodenal (**a**), rectal (**b**), and MEN-1-associated (**c**) pancreatic NET. Ghrelin (TRITC) is visualized as red and obestatin (FITC) as green. DAPI-stained nuclei are visualized as blue. Yellow color in the merged image indicates that ghrelin and obestatin are colocalized in the same cells with the same cytoplasmic distribution. Scale bars: 20 μm.

served in 36–68% of the cases [11, 13, 25]. The immunohistochemical results from our study show that, in total, 9 and 12% of the NET tumors are ghrelin and obestatin IR, respectively. Of the pancreatic NETs, obestatin immunoreactivity was found in 17% of the cases and ghrelin immunoreactivity in 14%. Our results are mainly similar when compared to those demonstrated by a recent study by Volante et al. [13]. In their study, only a minority of NETs expressed the peptides in occasional neoplastic cells. However, there are some differences between the two studies. We identified IR cells in typical lung NETs, while we were unable to verify any ghrelin/obestatin immunoreactivity in neoplastic cells in the parathyroid and ileocecal NETs. A plausible explanation for this discrepancy could be the limited number of cases included in every subgroup of NETs in both studies.

In accordance with our study, Volante et al. [13] also reported a heterogeneous protein distribution where more NETs expressed obestatin than ghrelin, and within the tumors the obestatin-IR cells represented only a fraction of the ghrelin-IR tumor cell population, suggesting that posttranslational mechanisms are the source of peptide processing. One explanation for the different protein distribution of the peptides could be the complex ghrelin gene locus. A revision of the human ghrelin gene structure has demonstrated novel exons and alternative splice variants. Transcripts from the ghrelin gene that do not code for ghrelin, but instead may encode C-ghrelin (which contains the coding region for obestatin), and a transcript coding only for obestatin were demonstrated, suggesting that ghrelin gene-derived peptides may also be produced independently of preproghrelin [26].

In previous studies, the expression of ghrelin and obestatin has been investigated in normal human tissues. The peptides are mainly expressed in endocrine cells of the upper gastrointestinal tract and pancreas, and are decreased in number distally. Rare ghrelin-IR cells have been displayed in the lung [12]. Double immunofluorescence studies have shown that the peptides are coexpressed by the same cells [8, 13].

In our previous report regarding the expression of the peptides in normal tissues, we were unable to detect expression in the esophageal, rectal, lung and thyroid samples [8]. In the present study however, ghrelin/obestatin-IR cells are detected in NETs originating from these organs. Ghrelin mRNA has been demonstrated in normal human esophagus and ghrelin-IR cells have been found in embryonic esophagus but not at the fetal stage. This suggests that ghrelin may be important during the first stages of development [12, 27]. Normal lung has previously been reported to show immunoreactivity for ghrelin in a small number of cells [12], and it is possible that the frequency of ghrelin/obestatin-producing cells in these organs is very low and therefore difficult to detect under normal conditions.

To our knowledge, this is the first report demonstrating the expression of obestatin in rectal NETs. Notably, ghrelin expression has also recently been reported in a presacral NET [28]. Our previous study of normal human tissues has shown that rectal mucosa does not contain ghrelin and obestatin [8]. A possible explanation for the observed ghrelin/obestatin immunoreactivity in rectal NETs could be that these peptides are expressed by multipotent neoplastic neuroendocrine stem cells. In accordance with other studies where obestatin and ghrelin are reported to be diffusely expressed in fetal thyroid but not in adult glandular tissue and then reexpressed in tumors [13, 29], we did not find any ghrelin/obestatin-IR cells in the normal thyroid in our previous study [8], whereas the present study shows immunoreactivity in medullary thyroid cancer.

In addition, ghrelin/obestatin-IR cells were demonstrated to be present in nesidioblastosis, a condition characterized by pancreatic islet cell hyperplasia. The ghrelin/obestatin-IR cells were located at the periphery of the islets, a location of mainly non-insulin-IR cells. The pathophysiological explanation for this expression remains to be elucidated. However, it might be explained by the general hyperplasia of the islets affecting all cells including the ghrelin/obestatin-expressing cells in the pancreas.

In response to ingestion of a standardized meal, blood obestatin levels in both the pancreatic NET patients and healthy individuals examined remained in the normal reference range. However, in the healthy individuals, the levels decreased after the meal test. This is in agreement with other studies, where a decrease in obestatin concentration after meal intake has been reported [30, 31].

In contrast, the obestatin concentrations remained largely unchanged in the patients during the entire test. The obestatin levels were also significantly higher in the patients than controls. It is plausible that the obestatin control/balance as well as its secretion are affected in some way due to the influence of the tumor. In a previous study, obestatin blood levels were measured in patients suffering from gastric endocrine tumors [10]. In that study, no grossly increased obestatin levels could be identified, and the circulating levels were consistently low. Probably, obestatin concentration in blood varies very little in general, and its function could mainly be of paracrine or autocrine character.

Furthermore, in accordance with other studies, no correlation between obestatin levels and BMI could be identified [30].

In summary, we have characterized a large panel of NETs with respect to obestatin and ghrelin protein expression, demonstrating that the peptides are mainly present in tumors of foregut origin as well as in rectal NETs. It is evident that only a minority of NETs expresses ghrelin and obestatin, and they are mainly limited to NETs originating from the same tissues that express the hormones under normal conditions. Our results using double immunofluorescence and consecutive sections show that the hormones are colocalized in the same cells when present in the same tumor. Furthermore, food intake had no effect on obestatin blood levels in patients with pancreatic NETs but caused a decrease in concentration in healthy controls. Although only a minority of NETs expresses ghrelin and obestatin, screening of NET patients may be relevant in the workup of these patients, especially of those patients with tumors originating from tissues that express ghrelin/obestatin in normal conditions.

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